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EFFECTS OF UNIALGAL AND BACTERIA-FREE CULTURES OF *GYMNODINIUM BREVIS* ON FISH AND NOTES ON RELATED STUDIES WITH BACTERIA

By Sammy M. Ray and William B. Wilson

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ABSTRACT

A series of experiments, seven with unialgal cultures and two with bacteria-free cultures, demonstrated the toxicity of *Gymnodinium brevis* Davis to six species of fish. Bacteria-free cultures were just as toxic as unialgal cultures. The cultures employed contained 0.6 to 4.8 million *G. brevis* per liter. Apparently the test fish were differentially sensitive to *G. brevis* cultures. In order of decreasing sensitivity, the test fish were *Membras vagrans*, *Mugil cephalus*, *Fundulus grandis*, *Mollienisia latipinna*, *Fundulus similis*, and *Cyprin lon variegatus*. The lethality of bacteria-free *G. brevis* cultures to fish clearly indicates that this dinoflagellate is the direct cause of the mass mortalities of fish and other marine animals that are associated with *G. brevis* blooms in the Gulf of Mexico.

Toxicity of *G. brevis* cultures does not depend on the presence of the living organisms. The more toxic portion of the culture passes through a millipore membrane whereas it is retained by filter paper. Such possible lethal factors as oxygen deficiency, suffocation due to clogging of gills with masses of organisms, and bacterial growth were eliminated experimentally, thereby establishing that *G. brevis* produces a toxic substance(s).

Two chromogenic marine bacteria, *Flavobacterium piscicida* Bein and an unidentified red-pigment-producing form from the west coast of Florida, were tested for toxicity to fish. The results of these tests are discussed.

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EFFECTS OF UNIALGAL AND BACTERIA-FREE CULTURES OF *GYMNODINIUM BREVIS* ON FISH, AND NOTES ON RELATED STUDIES WITH BACTERIA

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Association of the dinoflagellate *Gymnodinium revis* Davis with the mass mortality of marine nimals that occurs sporadically in the Gulf of Mexico is well established (Davis 1948; Galtsoff, 948 and 1949; Gunter et al., 1948; Wilson and Ray, 1956; Woodcock 1948; and others). Indirect evidence presented in these papers strongly upports the contention that *G. brevis* is the cause f fish kills, commonly referred to as red tides,[1] hen its concentration reaches the order of undreds of thousands to millions of organisms er liter—concentrations as high as 50 to 60 illion organisms per liter have been reported. his evidence includes (1) the presence of dead or ying fish in water containing such concentrations f *G. brevis*, (2) laboratory demonstration that ater containing great numbers of *G. brevis* is oxic to fish, and (3) demonstration that subances toxic to fish may be extracted from water nfested with *G. brevis*. Further evidence of a ore direct nature is provided by the demonration that unialgal cultures of *G. brevis* are toxic fish (Wilson and Collier, 1955).

Development of stock unialgal cultures of *. brevis* opened the way for the elucidation of this rganism's role in the mass mortality of marine nimals by making available an abundant supply f material for controlled experiments. Previous this development investigators were handiapped, since the suspected causative agent was unavailable for study except during outbreaks. Even then, their material was limited to raw samples from the infested waters which contained numerous other organisms. In addition, raw samples were probably held under conditions unsuitable for the survival of *G. brevis*.

The next approach to this problem was to obtain bacteria-free or pure cultures of *G. brevis*. This isolation is necessary to determine whether a cause-and-effect relation exists between *G. brevis* and the catastrophic fish kills. Furthermore, studies of such problems as the nutritional requirements of *G. brevis*, nature of the toxic substance, role of associated organisms, and effects of physical and chemical factors may be facilitated with the use of bacteria-free cultures since the uncertainty regarding the effects of associated bacteria would be eliminated.

These laboratory studies in coordination with field studies, will provide a better understanding of why the mass mortalities occur. Such knowledge will be helpful in predicting when and where outbreaks may be expected and in determining the feasibility of control measures.

This report presents the results of our studies on the effects of unialgal and bacteria-free cultures of *G. brevis* on fish as well as the effects of some bacteria isolated from unialgal cultures of this organism and from waters off the west coast of southern Florida.[2] Based upon the results of studies with bacteria-free cultures, we conclude that *G. brevis* produces the toxic substance(s) responsible for the mass mortality of marine animals associated with blooms of this organism in the Gulf of Mexico.

[1] The term "red tide" is generally applied to discolored sea water regardless causes or consequences; that is, the causes of the discolorations may vary om "blooms" of many different microorganisms to nonliving agents such iron compounds; and the mortality of animals, especially fish, may or may t be associated with such discolorations. To avoid confusion, we believe best to refrain from using this popular though nonspecific term in scientific iblications. If a popular name is used, we propose that the name "brevis d tide" be applied to the mass mortality of marine organisms associated th *Gymnodinium brevis*.

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[2] We are indebted to K. T. Marvin, Alice Kitchel, and Jean Gates for assistance in performing the experiments reported here and to E. L. Arnold and R. S. Wheeler for identifying the test fish.

PROCEDURES FOR TESTING STERILITY AND ENUMERATING ORGANISMS

Bacteria-free cultures were grown in the same medium prescribed by Wilson and Collier (1955) and were carried through several subcultures. After 10 months they showed no apparent diminution in vigor. Several media were used to establish sterility. All *G. brevis* cultures originated from a culture obtained from a sample collected in a bloom that occurred near the coast of Florida in September 1953. Details of the procedures for culturing *G. brevis*, and the methods used to obtain bacteria-free cultures will be presented in another paper.

STERILITY-TESTING MEDIA

Cultures of *G. brevis* used for transferring were tested for sterility in media prepared according to Spencer (1952): (1) peptone sea water (0.5%, bacto-peptone, 0.01%, $FePO_4$ dissolved in 75% aged sea water) and (2) peptone sea-water agar (peptone sea water plus 1.5% bacto-agar). These media as well as all other sterility-testing media subsequently described were dispensed in screw-cap tubes and autoclaved at 121° C. for 15 minutes.

We frequently used four other media similar to those employed by Droop (1954) for routine sterility testing. These media included (1) distilled-water liquid, (2) distilled-water agar, (3) sea-water liquid, and (4) sea-water agar. Our media contained the following substances: 0.5% dextrose, 0.1% Difco neopeptone, 0.4% bacto-beef extract, 0.5% bacto-yeast extract, 0.015% sodium acetate ($NaC_2H_3O_2.3H_2O$), and soil extract (2.0 ml./100 ml.). These substances (dextrose was often excluded) with and without bacto-agar (1.5%) were dissolved in both distilled water and 75% aged sea water to give the four combinations. Droop (1954) listed the substances used, but not the quantities. A personal communication (1956), however, revealed that his formula contained the organic substances in concentrations which were roughly 10 to 15 times less than the quantities we used. Furthermore, he included bacto-tryptone, which was not listed in his paper, whereas we used Difco neopeptone. Subsequent to the completion of the present studies, the absence of bacteria from several *G. brevis* cultures was confirmed with media of Droop's formulation and also with these media diluted to 10 percent.

Other media used to supplement the routine tests included (1) the sterility-test medium use by Sweeney (1954) containing 0.05% bact peptone, 0.0136% sodium acetate (NaC_2H_3O $3H_2O$), 0.0202% KNO_3, 0.00356% K_2HPO 0.00016% $FeCl_3.6H_2O$, and 0.000012% MnC $4H_2O$ dissolved in 75% aged sea water with an without bacto-agar (1.5%); (2) Spencer's peptor sea-water media supplemented with 0.1% bact yeast extract as employed in medium 2116 (Morita and ZoBell, 1955); (3) semisolid mediu composed of 0.075% trypticase (Baltimore Bi logical Laboratory), 0.075% bacto-pepton 0.075% bacto-yeast extract, 0.01% sodium aceta ($NaC_2H_3O_2.3H_2O$), and 0.2% Difco special (Nobl agar dissolved in aged sea water; (4) 1% bact peptone in aged sea water with and witho bacto-agar (1.5%), the medium used by Be (1954) to isolate and cultivate certain chrom genic bacteria found in Florida waters; an (5) Spencer's (1952) casein sea-water agar con posed of 0.05% bacto-peptone, 0.05% bact isoelectric casein, 0.05% soluble starch, 0.1 (v/v) glycerol, 0.02% K_2HPO_4, and 1.5% bact agar dissolved in 75% sea water.

Sterility tests for anaerobic bacteria were co ducted occasionally with three different medi (1) Bacto-fluid thioglycollate medium rehydrat with both distilled water and 75% aged s water; (2) the general anaerobic medium (slight modified) used for marine bacteria by Morita a ZoBell (1955) containing 0.5% bacto-pepton 0.1% bacto-yeast extract, 0.01% $FePO_4$, 0.1 sodium formaldehydesulphoxylate, and 0.0001 resazurin dissolved in 75% aged sea water wi and without bacto-agar (1.5%); and (3) an anae obic medium prepared by adding 0.01% sodiu thioglycollate to the semisolid medium describ in the previous paragraph. The melted, gener anaerobic agar medium was cooled to 40°–42° before addition of the test culture which w mixed by swirling the tube before the agar soli fied. After adding the test culture, sterile melt vaspar (50% vaseline and 50% paraffin) w poured into each tube of anaerobic mediu except the fluid thioglycollate medium, to exclu oxygen.

INOCULATION AND INCUBATION

All sterility tests, unless otherwise indicate were made with 1.0 ml. of test culture in 10.0 m

f medium. The agar media were inoculated in ıe following ways: (1) Pour-plate—mixing test ılture in a sterile Petri dish with melted medium ɔoled to 40°–42° C., (2) streak-plate—streaking .1 ml. of test culture on a freshly prepared plate, 3) stab culture—placing 0.1 ml. of test culture into ıedium in screw-cap tubes (20 mm. x 125 mm.), nd then stabbing an inoculating needle to the ottom, and (4) slant cultures—placing the test ılture on freshly slanted medium in screw-cap ıbes. Slant cultures were generally prepared for ıost routine tests. The agar plates were sealed ith masking tape to prevent desiccation and mold ɔntamination during incubation. Semisolid me-ium was inoculated by stabbing to the bottom ith a micropipette and then gradually releasing ıe inoculum as the pipette was slowly withdrawn.

We incubated the sterility-test cultures in the ark at 28°–30° C. for a minimum of 6 weeks efore discarding them as sterile. This tempera-ure level was selected since some of the bacteria ɔlated from the unialgal cultures of *G. brevis* ppear to grow more slowly at 24°–25° C.

On one occasion the sterility of several cultures ras tested in duplicate in various liquid and agar ıedia; the four methods for inoculating agar cul-ures were used. One set was incubated with lumination (175–300 ft.-c.) and temperature 24°–25° C.) the same as used for *G. brevis* cul-ures; the other set was incubated in the dark at 8°–30° C. After 6 weeks none of the cultures howed either visible colonies or cloudiness of any ort except an occasional mold or bacterial colony n the surface of a few streak- and pour-plates.

We attribute the occasional appearance of mold r bacterial colonies in our test cultures, especially n the surface at the periphery of streak- and our-plates, to contamination while the plates ere exposed by necessary manipulations. The osition of the colonies as well as the appearance f similar colonies on some control plates (unin-culated agar plates), which were treated in the ame manner as the test cultures, supports this onclusion. We rarely encountered accidental ontamination of sterility-test cultures contained n screw-cap tubes.

MISCELLANEOUS CHECKS FOR STERILITY

We consider that the medium used for culturing *. brevis* is unlikely to be suitable for the growth of hotosynthetic bacteria. Nevertheless, a few cultures were checked for such organisms. The checks were made with a medium developed by Dr. T. J. Starr of this laboratory for the isolation of marine nonsulfur purple bacteria. This medium is composed of 0.2% sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$), 0.05% $Na_2SO_3 \cdot 7H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.0001% $FeCl_3 \cdot 6H_2O$, 2.5% NaCl, 0.01% bacto-yeast extract, 0.01% sodium thioglycollate, and 1.5% Difco special (Noble) agar dissolved in double-distilled water. Just before inoculation the medium was melted, and sterile $NaHCO_3$ solution was added aseptically to each tube to give a 0.1% concentration and a final pH of 8.0. This medium was inoculated and treated in the same manner as previously described for the general anaerobic medium. These sterility-test cultures, which were incubated under the same light and temperature conditions used for *G. brevis*, showed no evidence of growth after 6 weeks.

Phase-contrast microscopic examination (× 970) of wet preparations of a few *G. brevis* cultures that were determined to be pure by cultural methods did not reveal any contaminating organisms. These examinations, conducted several months after the initial establishment of bacteria-free cultures, were performed to check for possible contaminants which might have maintained themselves in *G. brevis* culture medium after repeated subculturing, and yet had not grown in any of the sterility-test media employed.

BACTERIA ENUMERATION

Aerobic bacterial counts presented in the experiments to follow were estimated by plating serial dilutions of the test sample. The dilution water blanks (75% aged sea water) dispensed in 9-ml. amounts in screw-cap tubes were autoclaved at 121° C. for 15 minutes. Serial dilutions of 1:10; 1:100; 1:1,000; 1:10,000; and 1:100,000 were prepared of each sample to be counted. One ml. of each dilution and 10.0 ml. of melted Spencer agar, cooled to 40°–42° C., were mixed in a sterile Petri dish by gentle swirling before the agar hardened. Because of the possibility of low counts a plate was also prepared with 1.0 ml. of undiluted sample. After the agar hardened, the plates were sealed with masking tape and incubated in an inverted position for 4 to 7 days at 28°–30° C. Colonies were enumerated with the aid of a Quebec colony counter. Plates with either more than

300 or less than 30 colonies were not used in quantitative estimates except in a very few instances. The exceptions were in cases where either the most dilute plates contained more than 300 colonies or the undiluted plate contained less than 30 colonies.

The bacterial counts most likely represent minimal concentrations, because nutritional and environmental requirements of an entire bacterial population cannot be satisfied with any one medium or with a single set of incubation conditions. We made no attempt to enumerate anaerobic bacteria. All colonies except those with the typical appearance of molds were counted, therefore, any microorganisms producing bacterialike colonies were included in the counts.

Since we could not prepare pour-plates of all water samples immediately after collecting them, another possible source of error in the counts should be considered. Both quantitative and qualitative changes in the bacterial population may have occurred before some of the samples were plated, particularly those plated several hours or even days after collection. Immediately after collection all water samples were refrigerated (4° C.) until shortly before preparation of the plates. In most cases the storage period did n exceed 6 hours; however, this period varied co siderably in some experiments, especially tho in which several samples were counted. Cons quently, we have recorded the extremes of t storage period for each experiment.

DINOFLAGELLATE ENUMERATION

The concentration of *G. brevis* and other din flagellates was determined in two steps: (1) preliminary counting of 1.0-ml., 0.1-ml., and 0.0 ml. aliquots from a sample mixed by gently swirlin the tube (vigorous shaking frequently causes mar of the organisms to cytolyze) to determine samp size best for counting, and (2) counting 3 to aliquots of the quantity selected in the first ste The latter counts were averaged to obtain the *brevis* concentrations. The counts probably repr sent minimal levels because the organism tends disintegrate when manipulated. Because of th tendency, only one aliquot was withdrawn at time and it was counted immediately. A wid field stereoscopic microscope with a magnificatio of × 54 was used in making the counts.

EXPERIMENTS WITH UNIALGAL CULTURES OF *GYMNODINIUM BREVIS* AND OTHER DINOFLAGELLATES

Seven experiments testing the toxicity to fish of unialgal cultures of *G. brevis* and some other dinoflagellates were performed. All of these studies, even those which were preliminary, such as experiments 1 through 3, are presented because the details and results vary considerably in some cases. In some experiments only one test fish was used per container because either the available fish were too few or the containers were too small t accommodate more. Moreover, duplicate co tainers were not always used because of limit tions imposed by insufficient supply of either te fish or test materials. We have taken special ca to record all experimental details, some of whic may be of no significance, since they could prov of value to others in reviewing our work.

EXPERIMENT 1.—A Simple Test of the Toxicity of *Gymnodinium brevis* Cultures

This experiment was conducted to determine whether unialgal *G. brevis* cultures would kill fish. We used a 3½-week-old culture, replenished with fresh medium three times weekly, that contained 1.8 million organisms per liter. Sea water from a lagoon at the east end of Galveston Island, Texas—the locality where the test fish were collected—served as control material. The test materials were not aerated. One rough silversides (*Membras vagrans*), 3½ inches long, and one sailfin molly (*Mollienisia latipinna*), 2½ to 3 inches long, were placed in each of two 1-liter beakers—one containing sea water, the other *G. brevis* cultur The beakers were covered with polyethyle sheeting.

Membras vagrans survived only 4 minutes the *G. brevis* culture whereas this species survive 43 minutes in the sea water. *M. latipinna* di after 85 minutes' exposure to the *G. brevis* cultu and the fish in the sea water was alive when t experiment was discontinued 4 days later. A though the lethality of *G. brevis* cultures to fi is evident from these results, they do not nece sarily prove that a toxic substance is involved.

XPERIMENTS 2 and 3.—Comparison of Effects of *Gymnodinium brevis* and *Gymnodinium splendens* Cultures

The mere presence of numerous dinoflagellates ıay have been responsible for the toxicity of the nialgal culture used in experiment 1. To test ıis possibility, fish were subjected to unialgal *G. revis* and *G. splendens* cultures in experiments 2 nd 3. Experiment 2 was conducted under the ame conditions as experiment 1. A 4-week-old nialgal culture of *G. brevis* and a 10-week-old nialgal culture of *G. splendens* that contained 2.1 nd 2.8 million organisms per liter, respectively, ere tested for toxicity to *Mollienisia latipinna* 2½ to 3 in. long). Both cultures were replenished ith fresh medium three times weekly during the ıcubation period. Sea water from which the test sh were taken was used as control. One fish was laced in each of three test materials. The fish ı the *G. brevis* culture died after 47 minutes; the fish were alive at the close of the study 19 days later in the *G. splendens* culture and in sea water.

Experiment 3 duplicated experiment 2 in most respects, except that the cultures were a week older. At this time there were 2.0 million *G. brevis* and 2.6 million *G. splendens* per liter in the cultures. *M. latipinna* (2½ to 3 in. long) lived only 68 minutes in the *G. brevis* culture, but they were alive in the *G. splendens* culture and in sea water 3 days later when the experiment was discontinued.

The excellent survival of the fish in *G. splendens* cultures, in contrast with the lethality of *G. brevis* cultures, indicates that the latter cultures contained a toxic substance(s). Since the cultures of *G. brevis* were not pure, the toxic substance could have been produced by *G. brevis*, associated bacteria, or both.

EXPERIMENT 4.—Effects of Unialgal *Gymnodinium brevis* Cultures and Associated Bacteria

A series of experiments (4, 5, 6, and 7) was esigned mainly to determine whether *G. brevis* or s associated bacterial flora is responsible for the oxic effects of unialgal cultures to fish. If the acteria prove nontoxic under the same cultural onditions, one could reasonably assume that *G. revis* produces the toxic substance. Much of the alue with regard to the original purpose for conucting these experiments has been lost subseuent to the development of mass bacteria-free ultures of *G. brevis*. Bacteria-free cultures made possible to demonstrate experimentally that *G. revis* produces a fish-killing substance. The deails are presented later in this paper.

To obtain some of the test materials used in hese experiments (4, 5, 6, and 7), 20 liters of culure medium were prepared, 5 liters of which were laced in each of two Pyrex bottles (2½ gal.); nother Pyrex bottle (5 gal.) received the remainng 10 liters. Each bottle of medium was heated o 75° C. (5 to 6 hours' heating required) on three uccessive days to reduce the bacterial load. One f the 2½-gallon bottles (No. 1) was inoculated ith 10.0 ml. of a 6-week-old unialgal *G. brevis* ulture with a bacterial count of 8.1 million per nl. The other 2½-gallon bottle (No. 2) was eeded with 10.0 ml. of *G. brevis*-free inoculum in n attempt to culture the associated bacteria. This inoculum, having a bacterial count of 10.3 nillion per ml., was obtained by heating between 7°–39° C. for 30 minutes a portion of the same culture used to inoculate bottle 1. The 5-gallon bottle (No. 3) containing uninoculated medium was arranged so that bottles 1 and 2 could be replenished from this reservoir when culture materials were removed for toxicity tests and chemical analyses.

Samples were taken from the three bottles at irregular intervals during the first 25 days of incubation to follow the bacterial growth. The bacterial counts (table 1) of samples taken at 1-, 4-, 14-, and 25-day intervals from the unialgal *G. brevis* culture (bottle 1) and the *G. brevis*-free bacterial culture (bottle 2) were comparable except for the 4-day samples. The 4-day sample from the *G. brevis* culture had a bacterial count of 2.7 million per ml.—about 50 percent greater than that of the *G. brevis*-free bacterial culture. Some bacterial counts are questionable because of prolonged refrigeration of samples before preparation of the plates. They indicate, however, the relative number of bacteria in the three bottles at the various sampling intervals. Although bottles 1 and 2 apparently were inoculated with the same bacterial flora, we can only presume that the floras that subsequently developed in these bottles were qualitatively comparable.

Approximately 6 weeks after bottles 1 and 2 were inoculated, materials from these bottles and the reservoir (bottle 3), in addition to an 11-month-old unialgal *G. brevis* culture and centrifuged sea water, were used to conduct experiment

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TABLE 1.—*Experiments 4, 5, 6, and 7: Bacterial counts of samples taken from study bottles at irregular intervals*

[Bottles No. 1—unialgal *Gymnodinium brevis;* No. 2—*Gymnodinium brevis*-free bacteria; No. 3—reservoir (uninoculated)]

Incubation period	Number of bacteria per ml. (millions)	Remarks
Shortly before inoculation:		
Bottle No. 1	0.000070	Media cooled to room temperature after final heating before samples were collected; plates prepared shortly thereafter.
Bottle No. 2	0.000090	
Bottle No. 3	----------	
Shortly after inoculation:		
Bottle No. 1	0.014	Plates prepared shortly after samples were collected.
Bottle No. 2	0.018	
Bottle No. 3	----------	
1 day:		
Bottle No. 1	0.37	Samples refrigerated 5 days before plates were prepared.
Bottle No. 2	0.41	
Bottle No. 3	0.0015	
4 days:		
Bottle No. 1	2.7	Samples refrigerated 2 days before plates were prepared.
Bottle No. 2	1.8	
Bottle No. 3	0.26	
14 days:		
Bottle No. 1	2.0	Plates prepared 15 to 20 minutes after samples were collected.
Bottle No. 2	1.8	
Bottle No. 3	0.13	
25 days:		
Bottle No. 1	1.4	Samples refrigerated 2 days before plates were prepared.
Bottle No. 2	1.2	
Bottle No. 3	[1] 12.2	

[1] The value of 12 million bacteria per ml. for the reservoir appears to be excessively high when compared with the other counts obtained at either earlier or later intervals. With the exception of the presently considered value, the highest bacterial count obtained from the reservoir was 1.8 million per ml. (table 5, experiment 7).

4. One striped mullet (*Mugil cephalus*), 2½ to 3 inches long, was subjected to 750 ml. of each of the five different nonaerated test materials in 1-liter beakers. We observed the fish closely and recorded the time at which they began to show imbalance (distress time) and the time at which they showed no visible opercular movement (death time). Bacterial-count samples were obtained from each container before the fish was added. These samples were refrigerated 1 hour to 1½ hours before they were plated.

The results (table 2) of this 24-hour experiment show that the fish in the two unialgal *G. brevis* cultures died in 50 minutes and 2¼ hours. Two the three control fish survived considerably longe 7½ hours in the *G. brevis*-free bacterial culture ar the entire test period in the uninoculated cultu medium; however, the fish in the centrifuged s water died after 58 minutes. The early death this control fish was, perhaps, due to injury.

The bacterial count of 6.0 million per ml. f the *G. brevis* culture (bottle 1) in container 3 w five times greater than that for the *G. brevis*-fr bacterial culture (bottle 2) in container 4. Pri to this 6-week check the bacterial counts of the two cultures were comparable (table 1). Di parity in the bacterial counts for experiment necessitated additional studies in order to dete mine the toxicity agent in unialgal *G. brev* cultures.

TABLE 2.—*Experiment 4: Effects on* Mugil cephalus *unialgal* Gymnodinium brevis *cultures and of a* G. brev *free culture presumed to contain bacteria associated w this organism in unialgal cultures*

Container	Material in container	Distress time [1]	Death time [2]	Number of bacteria per ml. (millions)	Remarks
No. 1	Centrifuged sea water (Galveston beach).	0:50	0:58	0.0070	----------
No. 2	11-month-old *G. brevis* culture, 0.8 million organisms per liter.	0:05	0:50	10.2	Fresh medium added to *G brevis* culture occasionally
No. 3	6-week-old *G. brevis* culture (bottle 1), 0.6 million organisms per liter.	0:15	2:15	6.0	Do.
No. 4	6-week-old *G. brevis*-free bacterial culture (bottle 2).	([3])	([3])	1.2	Fresh medium added to bacterial culture occasionally
No. 5	6-week-old medium from reservoir (bottle 3).	([4])	7:35	0.080	----------

[1] Time (hr.:min.) required for fish to show first signs of imbalance.
[2] Time (hr.:min.) of cessation of opercular movement.
[3] Distress or death did not occur during the 24-hour test period.
[4] Not known.

EXPERIMENT 5.—Effects of Unialgal *Gymnodinium brevis* Cultures, Associated Bacteria, and Unialgal *Prorocentrum* sp. Cultures

In addition to testing the effects of *G. brevis* culture (bottle 1) and *G. brevis*-free bacterial culture (bottle 2), another dinoflagellate, *Prorocentrum* sp., was tested for toxicity to fish in the second experiment of this series. This organism was isolated from water samples taken in the lagoon, at Galveston, Texas. The materials in bottles 1 and 2 were 3½ months old at this time. Freshly collected sea water served as control. The four different materials, 2 liters of each in 4-liter beakers, were tested in duplicate f toxicity to *Mugil cephalus* (2½ in. long). O fish was tested in each container without aeratio Samples were collected from each container f bacterial counts before the fish was adde These samples were plated after 1½ to 5 hou refrigeration.

The test fish subjected to the *G. brevis* cultu died within an hour (29 and 47 minutes) where the fish in the other test materials lived a mi

mum of 8⅓ hours to a maximum of 24 hours—the duration of the experiment (table 3). The bacterial counts of both the *G. brevis* culture (bottle 1) and the *G. brevis*-free bacterial culture (bottle 2) had decreased since experiment 4 was conducted. Just as in experiment 4, however, the *G. brevis* culture had a much higher count—2.9 to 3.4 million bacteria per ml. in contrast with 0.20 to 0.23 million per ml. for the *G. brevis*-free culture.

One of the containers of *G. brevis* culture (6) used in experiment 5 was employed in a supplementary study to determine whether adding several fish to the same culture would affect its toxicity. Another phase of this study was to check the response of fish transferred to sea water after being subjected to *G. brevis* culture. Immediately after the fish in container 6 died (29 minutes after beginning of experiment 5) it was removed and the first of five additional *M. cephalus* were placed in this container. This fish succumbed after 21 minutes' exposure. After removing the dead fish, the second one was allowed to remain in container 6 for 15 minutes It was then transferred to sea water (container 1) where it died 12 minutes later. Exposures of 15 minutes and 7 minutes, respectively, in container 6, were required to kill the third and fourth fish. Each fish was removed from the container after it died. After 3 minutes' exposure in container 6, the fifth fish was removed to container 1 where it survived for 2¾ hours.

TABLE 3.—*Experiment 5: Effects on* Mugil cephalus *of unialgal cultures of* Gymnodinium brevis *and* Prorocentrum *sp. and* Gymnodinium brevis*-free culture presumed to contain bacteria associated with this organism in unialgal cultures*

Container	Material in container	Death time [1]	Number of bacteria per ml. (millions)	Remarks
No. 1	Freshly collected sea water (Galveston beach).	Between 10 and 22 hours.	0.0025	
No. 2	do	do	0.0030	
No. 3	3-month-old *Prorocentrum* sp. culture, 0.5 million organisms per liter.	[2]	0.59	Fresh medium added to *Prorocentrum* culture occasionally.
No. 4	do	Between 10 and 22 hours.	0.46	
No. 5	3½-month-old *G. brevis* culture (bottle 1), 1.9 million organisms per liter.	0:47	2.9	Fresh medium added to *G. brevis* culture occasionally.
No. 6	do	0:29	3.4	
No. 7	3½-month-old *G. brevis*-free bacterial culture (bottle 2).	Between 10 and 22 hours.	0.20	Fresh medium added to bacterial culture occasionally.
No. 8	do	8:20	0.23	

[1] Time (hr.:min.) of cessation of opercular movement.
[2] Death did not occur during the 24-hour test period.

Experiments 4 and 5 (tables 2 and 3) were inadequately controlled with regard to quantities of bacteria. Experiment 6 was performed in an attempt to correct this shortcoming.

EXPERIMENT 6.—Comparison of Toxicity of Unialgal Cultures of *Gymnodinium brevis* and *Prorocentrum* sp., and Effects of Heating and Filtration on Toxicity

Besides attempting to ascertain the source of the toxic substance in unialgal *G. brevis* cultures, this experiment included a study of the effects of heating and filtration on the toxicity of such cultures.

One month prior to conducting experiment 6 the remaining portion of the *G. brevis*-free bacterial culture (bottle 2) received an inoculum of unialgal *Prorocentrum* sp., which had proved nontoxic to *M. cephalus* in experiment 5 (table 3). This step was taken in an attempt to increase the bacterial concentration in bottle 2 to a level comparable to that in the *G. brevis* culture (bottle 1). Centrifuged sea water was used in addition to these two bottles of material, which were 4½ months old at this time. These three materials were tested in duplicate (containers 1 through 6). The test materials in all of these containers, except one container (2) of sea water, were sampled for bacterial counts just before the fish were added. These samples were refrigerated 20 minutes to 2½ hours before the plates were poured.

Five containers (7 through 11) of the test material were used to test the effects of heating and filtration on the toxicity of unialgal *G. brevis* cultures. Bacterial counts were not made for these materials because such information was not needed. A filtrate, which was prepared by passing *G. brevis* culture through filter paper (Whatman No. 42), was tested in duplicate. A single container of another test material consisted of the residues retained by the two filter-paper discs eluted in 2 liters of sea water. Two liters of *G. brevis* culture were passed through each disc.

Other test materials included single containers of *G. brevis* cultures that had been heated to 35° and 45° C.

Each of the 11 containers (4-liter beakers) received two common killifish (*Fundulus grandis*), 3 to 3½ inches long. The test materials, 2 liters in each container, were not aerated.

Only one of the four fish placed in each of the two control materials, sea water and *Prorocentrum* culture (bottle 2), failed to survive the 4-hour test period (table 4). On the contrary, the four fish subjected to the *G. brevis* culture (bottle 1) died before the end of the test period. The death times were 9, 16, 100, and 130 minutes. Again, however, the *G. brevis* culture (bottle 1) had a greater bacterial concentration than the material in bottle 2, in spite of the addition of *Prorocentrum* sp. a month earlier. The count for the former was 2.2 to 2.4 million bacteria per ml. in contras with 0.19 to 0.20 million per ml. for the latter The count for the material in bottle 2 is quit similar to that obtained for this bottle in experi ment 5 (table 3).

The fish lived 20 to 100 minutes in the *G. brevi* culture heated to 35° C. In the culture heate to 45° C. the death times were only 13 and 1 minutes. Three of the four fish exposed t filtrates of a *G. brevis* culture survived the experi mental period. The two fish subjected to th materials eluted from filter paper through whic *G. brevis* culture had passed died in 23 and 13 minutes. Filtration appears to reduce the toxicit of *G. brevis* cultures; however, other filterin methods must be tested before this effect can b established as a characteristic of filtration.

TABLE 4.—*Experiment 6: Effects on* Fundulus grandis *of unialgal cultures of* Gymnodinium brevis *and* Prorocentrum *sp., and effects of heating and filtration on toxicity*

[2 fish tested in each container]

Container	Material in container	Death time [1]	Number of bacteria per ml. (millions)	Remarks
No. 1	Centrifuged aged sea water (aged 1 month in dark)	3:10	0.12	
No. 2	do	(2)		
No. 3	4½-month-old *G. brevis* culture (bottle 1), 0.7 million organisms per liter.	0:09	2.4	Fresh medium added to *G. brevis* cultur occasionally.
No. 4	do	0:16 1:40 2:10	2.2	
No. 5 [3]	1-month-old *Prorocentrum* sp. culture (bottle 2), 0.9 million organisms per liter.	2:45	0.20	Fresh medium added to *Prorocentrum* cultur occasionally.
No. 6	do	(2)	0.19	
No. 7 [4]	Filtrate (No. 42 Whatman paper) of 9-month-old *G. brevis* culture, 1.2 million organisms per liter.	2:45		Fresh medium added to *G. brevis* culture oc casionally. Filtrate refrigerated 2 days.
No. 8	do	(2)		
No. 9	2 liters sea water (same as in containers 1 and 2) plus filter-paper discs used for containers 7 and 8. 2 liters of *G. brevis* culture passed through each disc.	0:23 2:10		Filter-paper discs refrigerated in dry beaker days, eluted with sea water just before fis were added.
No. 10	Same *G. brevis* culture used in preparing filtrates for containers 7 and 8 heated to 35° C., then cooled to room temperature.	0:20 1:40		No live *G. brevis* observed after culture wa heated.
No. 11	Same as container 10, except portion of culture heated to 45° C.	0:13 0:18		No live *G. brevis* observed after culture wa heated.

[1] Time (hr.:min.) of cessation of opercular movement.
[2] Death did not occur during the 4-hour test period.
[3] The *G. brevis*-free bacterial culture (bottle 2) was inoculated with unialgal *Prorocentrum* sp. 1 month prior to experiment.
[4] Another *G. brevis* culture was used for the heating and filtration studies because of insufficient culture in bottle 1.

EXPERIMENT 7.—Comparison of Toxicity of Unialgal *Gymnodinium brevis, Prorocentrum* sp., and *Gymnodinium* sp., and Effects of Filtration on Toxicity

The final toxicity study in this series (experiments 4–7) compared the effects of unialgal cultures of *Gymnodinium brevis*, *Prorocentrum* sp., and *Gymnodinium* sp. The two latter organisms were isolated from water samples taken in the lagoon at Galveston, Texas. *Gymnodinium* sp. is morphologically similar to the cultured forms of *G. brevis* originally isolated from Florida waters. A portion of the experiment was to determine whether passage of *G. brevis* cultures through millipore membrane would reduce toxicity as di their passage through filter paper.

Striped mullet (*Mugil cephalus*) and variegate minnows (*Cyprinodon variegatus*) were used a test fish. The mullet (3 to 4 in. long) were main tained in aerated aquariums about 24 hours befor beginning the experiment. The minnows (abou 1¼ in. long), collected 3 days prior to beginnin

of the experiment, were kept in a nonaerated aquarium since this species survives well without aeration.

Five of the seven different test materials included in this study were tested in duplicate. These materials consisted of two different unialgal *G. brevis* cultures (containers 1 and 2, 3 and 4); of *Gymnodinium* sp. (containers 5 and 6); *Prorocentrum* sp. (containers 7 and 8); and of culture medium from bottle 3 (containers 9 and 10). Also included was a filtrate prepared by passing 1 liter of *G. brevis* culture through a millipore membrane (container 11) and the residues retained with this membrane eluted in 1 liter of culture medium from bottle 3 (container 12). The millipore membrane (HA) retains particles as small as 0.5 micron. Each of the 12 containers (2-liter beakers) received approximately 1 liter of test material that was not aerated.

Before adding the fish, bacterial samples were taken from two containers of *G. brevis* culture (1 and 2) and one container of culture medium (9). These containers were arbitrarily selected in order to compare the bacterial counts in some of the containers before the fish were added with those counts obtained after death of the test fish. The samples for bacterial counts were refrigerated from 3 to 3⅔ hours before the preparation of pour-plates. All containers with dinoflagellates (1 through 8) as well as the filtrate of the *G. brevis* culture (container 11) were sampled for counts of these organisms just before the fish were added. These counts were completed within 3 hours after collection of samples.

One *M. cephalus* was placed in each of the 12 containers. Each *M. cephalus* was removed from its container shortly after death and a *C. variegatus* was added. The fish were not introduced simultaneously, since the *M. cephalus* were rather large for the containers.

Immediately after the *M. cephalus* died in the containers (1, 2, and 9), that were initially sampled for bacterial counts, these containers were again sampled for such counts. Such samples were also taken from one of each of the remaining duplicate test materials (containers 3, 5, and 7) and from the millipore filtrate (container 11) following death of the *M. cephalus*. Pour-plates were prepared with these samples after 4 to 6½ hours' refrigeration, except that the sample from container 7 was stored only 1⅓ hours.

The results (table 5) of this 27-hour experiment show that all of the *M. cephalus* subjected to either *G. brevis* culture or filtrate and residues of such a culture died in less than an hour. The death times varied from 14 to 53 minutes. *M. cephalus* in the uninoculated culture medium lived approximately 2⅓ and 3 hours. The bacterial count of 1.8 million per ml. of this culture medium (bottle 3) was higher than the 1.0 to 1.5 million

TABLE 5.—*Experiment 7: Effects of unialgal cultures of* Gymnodinium brevis, Prorocentrum *sp., and* Gymnodinium *sp. on* Mugil cephalus *and* Cyprinodon variegatus *and effects of filtration on toxicity of unialgal cultures*

Container	Material in container	*Mugil cephalus*		*Cyprinodon variegatus*		Number (in millions) of—	
		Distress time [1]	Death time [2]	Distress time [1]	Death time [2]	Dinoflagellates per liter [3]	Bacteria per ml.[4]
No. 1	5-week-old *G. brevis* culture	0:39	0:49	4:38	5:20	1.3	1.5 (1.3)
No. 2	do	0:10	0:16	Not known	7–8 hours	1.3	1.0 (1.1)
No. 3	1-year-old *G. brevis* culture, fresh medium added 6–8 weeks prior to use.	0:10	0:16	2:39	2:42	1.2	(1.1)
No. 4	do	0:09	0:17	2:42	6:04	1.0	
No. 5	6-week-old *Gymnodinium* sp. culture	0:32	1:09	2:10	2:23	1.4	(0.90)
No. 6	do	0:22	0:46	2:23	2:42	1.1	
No. 7	8-month-old *Prorocentrum* sp. culture, fresh medium added approximately weekly.	Not known	6:52	(5)	(5)	1.0	(3.3)
No. 8	do	Not known	6:51	(5)	(5)	0.9	
No. 9 [6]	6-week-old culture medium (uninoculated) from reservoir (bottle 3).	2:13	2:20	(5)	(5)		1.8 (2.1)
No. 10	do	2:54	3:05	(5)	(5)		
No. 11	Millipore filtrate of portion of same culture used in containers 3 and 4.	0:06	0:14	3:26	4:30	0	(0.0060)
No. 12	Millipore membrane used to obtain filtrate in container 11 eluted in 1 liter culture medium from bottle 3 (same as used in containers 9 and 10).	0:48	0:53	(5)	(5)		

[1] Time (hr.: min.) required for fish to show first signs of imbalance.
[2] Time (hr.: min.) of cessation of opercular movement.
[3] All samples for dinoflagellate counts were collected from the containers just before introduction of the fish.
[4] Samples for the first bacterial count listed for each container were collected just before introduction of the *M. cephalus;* samples for the second count, in parentheses, were taken immediately after the *M. cephalus* died.
[5] Death or distress did not occur during the 27-hour test period.
[6] Original supply of medium in reservoir (bottle 3) became exhausted and it was renewed about 6 weeks prior to experiment.

per ml. obtained for *G. brevis* cultures. *M. cephalus* in the *Prorocentrum* culture survived nearly 7 hours. Those exposed to *Gymnodinium* sp. died after 46 and 69 minutes.

Cyprinodon variegatus survived considerably longer than *M. cephalus* in all test materials. In *G. brevis* cultures the death times for *C. variegatus* varied from 2⅔ to 7–8 hours. This species survived the 27-hour test period in the two control materials (the culture medium and the *Prorocentrum* culture). *C. variegatus* lived about 2⅓ and 2⅔ hours in *Gymnodinium* sp. culture.

M. cephalus lived only 14 minutes in the millipore filtrate of the *G. brevis* culture, in contrast with 53 minutes in material eluted from the millipore membrane. Likewise the filtrate was more toxic than the residues to *C. variegatus*: the fish lived 4½ hours in the filtrate, whereas the fish in the residues survived the test period.

RESULTS OF EXPERIMENTS WITH UNIALGAL CULTURES

The fish subjected to unialgal cultures of *G. brevis*, with the exception of one fish, died more rapidly than those exposed to control materials in the seven experiments considered in this section. The greater survival of the control fish demonstrates that unialgal cultures of this organism are toxic to the five species of fish tested (*Membras vagrans*, *Mugil cephalus*, *Fundulus grandis*, *Cyprinodon variegatus*, and *Mollienisia latipinna*). Indeed, the rapidity with which the fish succumbed in some of the cultures emphasizes the toxicity of unialgal *G. brevis* cultures. For example, the minimum death times for some of these fish were 4 minutes for *M. vagrans* (only 1 fish tested), 14 to 16 minutes for *M. cephalus*, and 9 to 16 minutes for *F. grandis*. However, all five species did not show such extremes of sensitivity to *G. brevis* cultures. The minimum death times for *C. variegatus* were 2⅓ to 2⅔ hours.

The concentration of *G. brevis* in the cultures used for the seven experiments varied from 0.6 to 2.1 million per liter. Since these cultures were not free of bacteria, such organisms possibly contributed to their toxicity.

The results of a preliminary study suggest that the survival period after exposure to *G. brevis* depends on the length of exposure and that fish subjected for just a few minutes may not recover when transferred to sea water. *M. cephalus*, subjected to unialgal *G. brevis* for 3 and 15 minutes and then transferred to sea water, lived for 165 and 12 minutes, respectively (experiment 5).

Moreover, there is some suggestion that *G. brevis* cultures may become more toxic with each subsequent addition of test fish. For example, in experiment 5, the death times of each of four *M. cephalus* added in succession to the same culture decreased progressively from 29 to 7 minutes. We do not know the reason(s) for this apparent increase in toxicity. Since the test materials were not aerated, possibly the oxygen content was progressively lowered by the test fish and the death times decreased thereby.

The toxicity of unialgal *G. brevis* cultures does not depend on the presence of living organisms. Cultures heated to 35° and 45° C. were no less toxic than the untreated ones. The removal of *G. brevis* from a culture by millipore filtration did not reduce the toxicity. The relative toxicity of a filtrate appears to be dependent upon the type of filter membrane employed. A paper membrane (Whatman No. 42) apparently retains the more toxic portion of the culture.

Attempts to determine whether *G. brevis* or its associated bacterial flora produces the toxic substance were not entirely successful. *G. brevis*-free cultures presumed to contain the bacterial flora associated with unialgal cultures of this organism were not toxic to the test fish. However, when these experiments were performed, the bacterial counts of these cultures were considerably lower than those of the *G. brevis* cultures. Nevertheless, a culture of presumed associated bacteria with a count of slightly more than a million per ml. proved nontoxic to *M. cephalus*. The bacterial count of this culture in experiment 4 (table 2) was comparable to those of the unialgal cultures that were toxic to *M. cephalus* and *C. variegatus* in experiment 7 (table 5). Furthermore, in experiment 7 uninoculated culture medium containing about 2 million bacteria per ml., which was slightly higher than the counts of two different *G. brevis* cultures, was not toxic to the test fish.

Unialgal cultures of three species of dinoflagellates (*Gymnodinium splendens*, *Gymnodinium* sp., and *Prorocentrum* sp.) isolated from the lagoon, Galveston, Texas, were tested for toxicity to fish. Two species proved nontoxic and one toxic. The nontoxic forms, *G. splendens* and *Prorocentrum* sp., were used in concentrations comparable to

and, in some cases, even exceeding those of the toxic *G. brevis*. In fact, *M. cephalus* survived considerably longer in the *Prorocentrum* culture than in the control material (uninoculated culture medium) in experiment 7 (table 5). *Prorocentrum* possibly aided survival of the fish by liberating oxygen since the test materials were not aerated. *Gymnodinium* sp. was toxic to *M. cephalus* and *C. variegatus* (table 5). In view of these results and since this organism in culture is morphologically similar to *G. brevis*, we tentatively consider the Galveston *Gymnodinium* to be *G. brevis*. The Galveston *Gymnodinium* was observed only three times (all within the same week) although samples were collected from the lagoon three times weekly during an 11-month period. The concentrations in the lagoon samples varied from 1,000 to 60,000 organisms per liter.

EXPERIMENTS WITH BACTERIA-FREE CULTURES OF *GYMNODINIUM BREVIS*

Mass bacteria-free cultures of *G. brevis* were established following the completion of experiment 7. The two experiments, 8 and 9, to follow were the first toxicity tests to be conducted with pure cultures. The importance of these studies lies in the fact that the observed effects can be attributed to *G. brevis* with certainty since no other organisms were present during the incubation period. Substantiation of the toxicity of unialgal *G. brevis* with bacteria-free *G. brevis* should establish the existence of a cause-and-effect relation between blooms of this organism and associated mass mortality of marine animals. The experiments with bacteria-free cultures were more refined in several respects than those with unialgal cultures. In addition to aerating most of the test materials, such factors as temperature, dissolved oxygen, salinity, and pH were determined.

EXPERIMENT 8.—Effects of Unialgal and Bacteria-Free *Gymnodinium brevis* Cultures

The first mass bacteria-free cultures of *G. brevis* were tested for toxicity to striped mullet (*Mugil cephalus*) and variegated minnows (*Cyprinodon variegatus*). *C. variegatus* (1¼ to 1½ in. long) were maintained in aerated aquariums for 5 days and *M. cephalus* (1 to 1¼ in. long) were brought into the laboratory the night preceding the experiment. One liter of distilled water was placed in each 2-liter beaker, containers for the various test materials, 5 days before commencing the experiment so that the aeration equipment could be tested and adjusted. The water was discarded and the beakers received no further treatment before introduction of the various test materials. The material in 12 of the 14 containers received gentle aeration continuously from a small aerator; the main air line from the aerator was equipped with a nonabsorbent cotton filter. To preclude possible excessive oxygen demand by *G. brevis*, light was provided continuously with two fluorescent lamps equipped with two 18-inch, 15-watt daylight tubes. The experimental setup is shown in figure 1.

Six different bacteria-free cultures (containers 3, 4, 6, 7, 9, 10, 12, and 13) and four batches of sterile control material (containers 5, 8, 11, and 14), all of which had incubated for a month, were employed in this study (table 6). The control material consisted of uninoculated culture medium otherwise subjected to the same conditions as the inoculated medium. The sterility of the *G. brevis* cultures and control materials was established by inoculating routine sterility-test media with samples withdrawn from the culture vessels shortly before these materials were dispensed into the experimental containers. Duplicate containers (3 and 4, 6 and 7) of two of the six bacteria-free cultures were set up to compare the survival of test fish in aerated (containers 3 and 6) and nonaerated (containers 4 and 7) cultures. A year-old unialgal *G. brevis* culture (container 1), which was replenished with fresh medium about 4 days previously, was used to compare the effects of unialgal and bacteria-free cultures. Aged sea water (container 2) served as control material for the entire experiment. The volume of test material placed in each container varied from 750 to 1,000 ml.

Before adding the fish, samples were taken from four containers (3, 6, 9, and 12) of bacteria-free *G. brevis* culture and two containers (5 and 11) of uninoculated culture medium for bacterial counts. Also at this time the nine containers with *G. brevis* were sampled for enumeration of this organism.

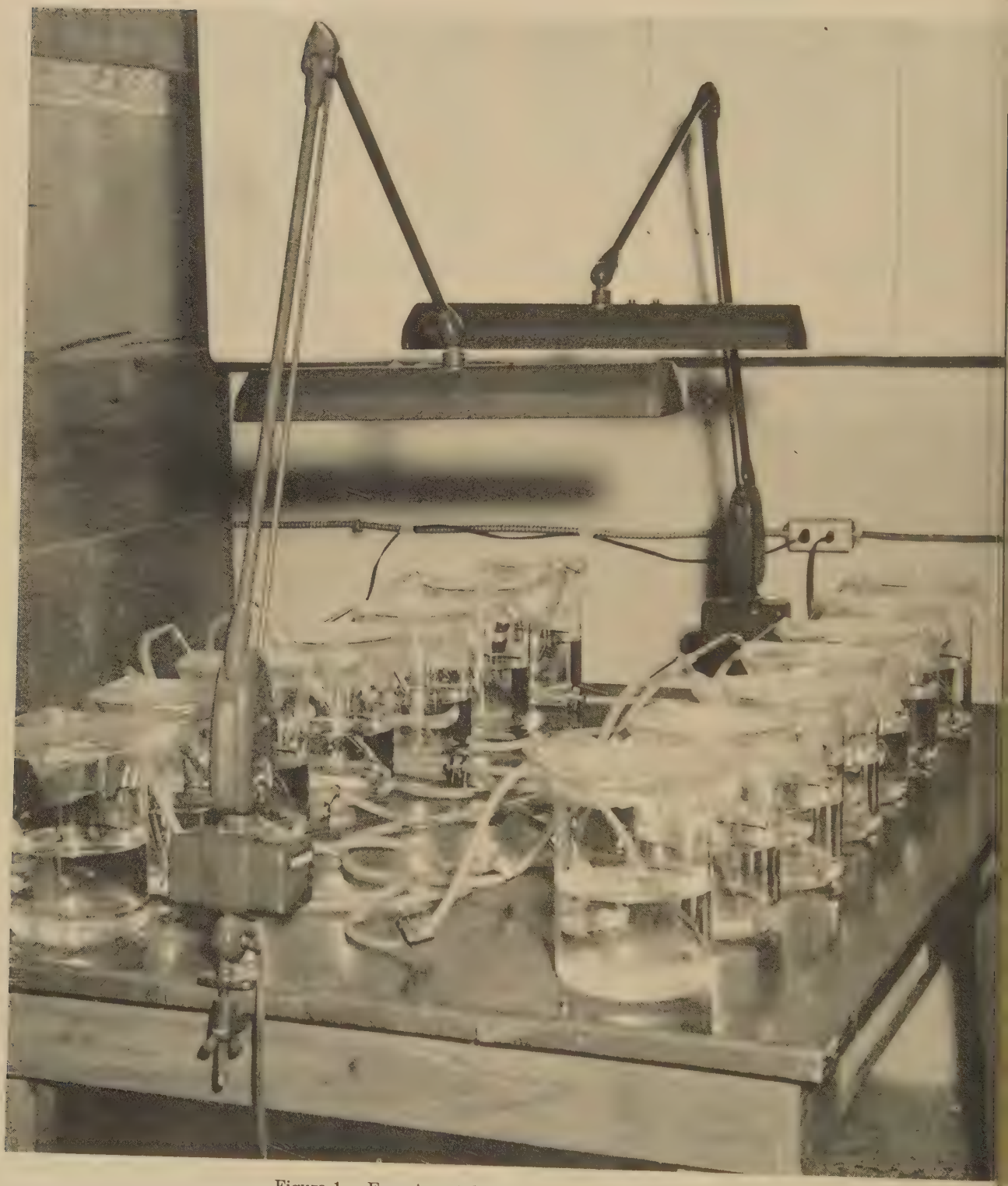

Figure 1.—Experimental setup used in experiment 8.

TABLE 6.—*Experiment 8: Effects of bacteria-free and unialgal cultures of* Gymnodinium brevis *on* Mugil cephalus *and* Cyprinodon variegatus

[All containers received 2 fish of each species except No. 2, in which 3 *M. cephalus* were tested]

Container	Material in container [1]	*Mugil cephalus*		*Cyprinodon variegatus*		Number (in millions) of—	
		Distress time [2]	Death time [3]	Distress time [2]	Death time [3]	*Gymnodinium brevis* per liter [4]	Bacteria per ml. [4]
No. 1	1,000 ml. unialgal *G. brevis* culture, pH 8.0	1:06 1:07	1:13 1:13	5:55 Not known	6:17 6:47	3.4 (2.4)	--- (3.5)
No. 2	1,000 ml. aged sea water	(5)	(5)	(5)	(5)	---	(5.0)
No. 3	1,000 ml. bacteria-free *G. brevis* culture	0:13	0:19	3:10	3:18	4.1	0.00025
No. 4	1,000 ml. of same culture used in container 3, but not aerated.	0:19 1:34	0:29 1:44	4:55 5:42	5:33 5:47	(3.7) 3.1	(0.033) ---
No. 5	1,000 ml. sterile culture medium, pH 7.6—control for 3 and 4.	1:39	1:44	5:44 6:50	5:56 29:48	(2.0) ---	(0.075) 0.0010
No. 6	800 ml. bacteria-free *G. brevis* culture	(5) 1:07	(5) 1:47	(5) 7:20	(5) 7:47	--- 4.8	(21.0) 0.020
No. 7	800 ml. of same culture used in container 6, but not aerated.	1:38 0:28	1:48 0:34	7:22 5:10	7:50 6:05	(2.6) 4.0	(0.30) ---
No. 8	800 ml. sterile culture medium, pH 7.6—control for 6 and 7.	0:28 (5)	1:56 (5)	6:31 (5)	7:02 (5)	(3.2) ---	(0.60) ---
No. 9	1,000 ml. bacteria-free *G. brevis* culture, pH 7.7	0:10	0:15	3:40	3:51	3.2	0.0070
No. 10	do	0:12	0:25	4:55	4:58	(2.7)	(0.075)
No. 11	1,000 ml. sterile culture medium, pH 7.7—control for 9 and 10.	0:13 0:13 (5)	1:09 1:12 (5)	3:21 3:48 (5)	3:44 5:54 (5)	3.5 (2.4) ---	--- --- 0.025
No. 12	750 ml. bacteria-free *G. brevis* culture	4:18	4:24	6:07	27:17	3.4	(7.0) 0.0050
No. 13	do	4:32 2:16	4:44 2:26	31:27 7:14	[6] 31:57 7:58	(1.8) 2.3	(24.0) ---
No. 14	750 ml. sterile culture medium—control for 12 and 13.	2:19 (5)	2:41 (5)	7:40 (5)	15:08 (5)	(1.5) ---	--- ---

[1] Determinations of pH made on samples withdrawn directly from culture vessel. All containers aerated except as noted.

[2] Time (hr.:min.) required for fish to show first signs of imbalance.

[3] Time (hr.:min.) of cessation of opercular movement.

[4] The first *G. brevis* and bacterial counts listed for each container were obtained from samples collected before introduction of the fish. Samples for second counts, in parentheses, were taken immediately after the death of last fish in container, except for two *G. brevis* counts (containers 12 and 13) and four bacterial counts (containers 2, 5, 11, and 12); the former were taken about 8 hours and the latter 30 to 31½ hours after start of experiment.

[5] Distress or death did not occur during the 31½-hour test period.

[6] The last fish (*C. variegatus*) in container 12 died about 30 minutes after close of experiment.

The samples taken for the initial bacterial counts were refrigerated from 1 hour to nearly 3 hours before preparation of the pour-plates. Most of the *G. brevis* counts were completed within a few minutes to an hour after withdrawal of the samples. A few samples, however, stood for a maximum of approximately 3 hours.

Each of the 14 containers received two *M. cephalus* and two *C. variegatus* except the container (2) of sea water, which received three *M. cephalus*. Each fish was removed from the container shortly after it died so that test materials would not become excessively fouled by decomposing fish.

After commencing the experiment, samples were again taken for *G. brevis* and bacterial counts. Seven of the containers with *G. brevis* were sampled for counts of this organism immediately after the death of the last fish in the container. Two other containers (12 and 13) of *G. brevis*, in which the last fish in the container survived beyond 8 hours, were sampled about 8 hours after beginning the experiment. The second set of samples for bacterial counts was taken either immediately after the death of the last fish in the container or after 30 to 31½ hours, provided that one fish survived the test period (31½ hours). The 10 containers sampled for bacterial counts included all of those that were sampled for such counts initially (containers 3, 5, 6, 9, 11, and 12), the container (1) of unialgal *G. brevis*, the container (2) of sea water, and the two containers (4 and 7) of nonaerated *G. brevis* cultures. These samples were plated after 15 to 90 minutes' refrigeration.

Following the collection of bacterial and *G. brevis* samples, all test materials were sampled for dissolved oxygen, pH, and salinity determinations (table 7). Aeration of each container (if aerated) was discontinued just before collection of the samples, which were taken either immediately after the death of the last fish in the container or near the end of the experimental period if at least one test fish survived. Seven containers (2, 5, 8, 11, 12, 13, and 14), those in which at least one fish survived beyond 7 hours, were also sampled for pH determinations 7 to 7½ hours after start of the study. During this 31½-hour experiment, room temperature varied from 20° to 24.5° C.

TABLE 7.—*Experiment 8: Dissolved oxygen, temperature, salinity, and pH data for test materials*

[See table 6 for materials in containers]

Container	Dissolved oxygen		Temperature [2] (° C.)	Salinity (0/00)	pH [3]	Time [4] (hours)
	P. p. m.	Saturation [1] (%)				
No. 1	6.77	89.4	22.8	29.84	7.5	7
No. 2	6.10	85.2	23.6	35.93	(7.6) 7.4	30¾
No. 3	6.70	89.3	21.9	32.73	7.3	5½
No. 4	3.38	45.0	22.1	32.72	7.2	6
No. 5	5.69	77.8	23.8	32.66	(7.2) 7.2	30⅓
No. 6	6.02	81.7	23.0	33.43	7.3	7½
No. 7	4.18	56.7	22.8	33.86	7.4	7
No. 8	5.59	76.8	23.8	33.44	(7.2) 7.2	30¾
No. 9	6.48	86.1	21.7	33.80	7.4	5
No. 10	6.84	91.5	22.4	32.71	7.4	6
No. 11	6.62	90.8	23.7	32.97	(7.3) 7.3	31
No. 12	6.60	90.6	23.9	32.69	(7.3) 7.4	31
No. 13	6.34	86.0	23.3	32.71	(7.3)	15
No. 14	6.10	83.7	23.8	32.97	(7.1) 7.2	31¼

[1] Percentage of saturation in relation to sea water of the given temperature and salinity in equilibrium with normal dry atmosphere.
[2] Temperature of material in container at time dissolved oxygen was determined.
[3] Values in parentheses were determined 7 to 7½ hours after start of experiment.
[4] The approximate time that elapsed between start of experiment and collection of samples for analyses.

Only 1 of the 32 fish subjected to initially bacteria-free cultures survived the 31½-hour test period; all except 3 (*C. variegatus*) died within 8 hours (table 6). The lone fish (*C. variegatus*) surviving the experimental period succumbed about 30 minutes later. On the contrary, only 1 of the 21 fish exposed to control materials of sea water and initially sterile culture medium failed to survive the test period. This fish (*C. variegatus*) died after nearly 30 hours in culture medium. The death times of *M. cephalus*, which varied from ¼ hour to 4¾ hours, were considerably less than were those of the *C. variegatus*, 3¼ to nearly 32 hours. Fifty percent (16) of the test fish in the bacteria-free cultures died earlier than those (4) in the unialgal culture; the death time in this culture was about 1¼ hours for *M. cephalus* and 6¼ and 6¾ hours for *C. variegatus*. The test fish survived considerably longer in the nonaerated than in the aerated *G. brevis* culture in one instance; in the other case the opposite occurred although the difference was less marked.

The bacterial counts of the initially bacteria-free *G. brevis* cultures sampled before adding the test fish varied from 250 to 20,000 per ml. The rather high initial bacterial counts are attributed to the prolonged standing (5 days) of the distilled water in the experimental containers while the aeration equipment was being tested and adjusted. The counts obtained from these cultures following the death of the last fish in the container (5 to 8 hours later) varied from 33,000 to 600,000 bacteria per ml. A count of 24 million bacteria per ml. was obtained after 31½ hours from the initially bacteria-free *G. brevis* culture in which one fish survived the test period. The first bacterial counts for two containers of initially sterile culture medium were 1,000 (container 5) and 25,000 (container 11) per ml. When these containers were sampled again near the end of the 31½-hour test period the bacterial count had increased to 21 million (container 5) and 7 million (container 11) per ml.

The results of experiment 8 show clearly that bacteria-free *G. brevis* cultures are toxic to fish. Nevertheless, we desired to confirm these results by using test materials with greatly reduced initial and terminal bacterial counts.

EXPERIMENT 9.—Effects of Bacteria-Free and Unialgal *Gymnodinium brevis* Cultures, and Effects of Filtration on Toxicity

The second study with bacteria-free *G. brevis* differed somewhat from the first one (experiment 8). In experiment 9, the initial bacterial contamination of test materials by containers and aeration equipment was reduced, the effects of two methods of filtration on the toxicity of bacteria-free cultures were studied, and the sensitivity of two size groups of mullet (*Mugil cephalus*) to *G. brevis* cultures was compared. The experimental setup was the same as for experiment 8 (fig. 1) except that more containers and a larger air pump were used.

We employed three precautions to reduce the initial bacterial contamination of the test materials. One precaution was to heat the experimental containers (2-liter beakers) in a hot air oven at 150°–160° C. for 2 hours and allow them to cool overnight in the oven. The containers were removed from the oven shortly before the test materials were added. Secondly, the aeration apparatus was autoclaved for 15 minutes at 15 pounds pressure. Before sterilization this apparatus was assembled and packaged so that a glass air-delivery tube could be inserted into each of the 18 containers without handling the portion that contacted the test materials. All air pumped

nto the containers passed through a nonabsorbent otton filter installed in the main air line from he pump. The third safeguard against excessive acterial contamination was to place the test fish i autoclaved 85-percent aged sea water for everal minutes before their transfer to the experiental containers. This concentration of sea ater is about the same as that in the culture edia to which the fish were exposed.

Three different, month-old bacteria-free *G. brevis* ultures, two batches of sterile culture medium of he same age, a 10-week-old unialgal *G. brevis* ulture, and autoclaved 85-percent aged sea water onstituted the test materials. One liter of test aaterial was placed in each of the 18 containers. ince the test materials were aerated more vigorusly in this study, the increased air flow made qualizing the degree of aeration in each container ifficult and the agitation of the test materials robably varied more.

One portion of the experiment (containers 1 hrough 8) was designed to compare the effects of illipore (HA membrane) and paper (Whatman o. 40) filtration on the toxicity of one of the acteria-free *G. brevis* cultures. One batch of the terile culture medium treated in the same manner s the *G. brevis* culture served as control material. he residues of millipore and paper filtration of oth the *G. brevis* culture and the sterile culture edium were each eluted in 1 liter of sterile culture edium to obtain four of the eight test materials sed in this phase of the study. The test materials or the remaining portion of this experiment (conainers 9 through 18) consisted of duplicate ontainers of two different bacteria-free *G. brevis* ultures, sterile culture medium, autoclaved 85ercent aged sea water, and a unialgal *G. brevis* ulture. The distribution of the test materials nd numerical designation of the containers are resented in table 8.

Samples were taken for bacterial and *G. brevis* ounts immediately after the test materials were lispensed. All containers of unfiltered bacteriaree *G. brevis* culture (9, 10, 11, and 12) and of nfiltered culture medium (13 and 14), in addition o a container of sea water (15) and a container of nialgal *G. brevis* culture (17), were sampled for acterial counts. The containers of filtrates or esidues were not sampled because such counts ere not needed. These samples were refrigerated ½ to 6½ hours before being plated. Samples for *G. brevis* counts were obtained from the containers with filtrates of *G. brevis* culture (1 and 2) and bacteria-free *G. brevis* (9, 10, 11, and 12). The *G. brevis* concentration of the unialgal culture used in containers 17 and 18 was ascertained by withdrawing a sample from the culture before dispensing it. The *G. brevis* samples were counted between 1½ to 2⅔ hours after collection.

Each container received four fish: three small mullet (1 to 1¼ in. long) and one large mullet (4½ to 5½ in. long). The small mullet were maintained in aerated aquariums overnight and the large mullet were used within a few hours after they were collected. Since the volume (1 liter) of test material was somewhat small for the large mullet, they thrashed about vigorously and possibly injured some of the smaller test fish.

Excepting the filtrates of *G. brevis* cultures (containers 1 and 2), the bacteria and *G. brevis* were again enumerated for those containers for which such counts were made initially. The second set of bacterial samples from the five containers with *G. brevis* (9, 10, 11, 12, and 17) was plated after 4¾ to 5¼ hours' refrigeration whereas those samples from the containers with culture medium (13 and 14) and sea water (15) were stored only ⅔ hour to 1¼ hours. The second set of *G. brevis* counts was completed between 1 hour and 2 hours after the samples were collected. In addition, samples for pH and salinity determinations were taken from all 18 containers. Bacterial, *G. brevis*, pH, and salinity samples were taken either shortly after the death of the last fish in the container or at the end of the test period (24 hours) provided that one fish in the container survived, except for some pH and salinity samples as noted under Remarks in table 8. The room temperature varied from 22° to 25° C. during the 24-hour test period.

The fish subjected to bacteria-free cultures as well as filtrates and residues of such cultures died more rapidly, for the most part, than those exposed to the control materials (table 8). The survival rate among the control fish, especially small *M. cephalus*, was not good. Fish of this size group died quickly in one batch of sterile culture medium (containers 13 and 14). The death times, varying from 25 minutes to 2⅔ hours, for the small mullet in these two containers were comparable to the death times, varying from 15 minutes to 2½ hours, for the same-sized fish in unfiltered bacteria-free

TABLE 8.—*Experiment 9: Effects on two size groups of* Mugil cephalus *of bacteria-free and unialgal* Gymnodinium bre *cultures, and effects of filtration on the toxicity of bacteria-free cultures*

[1 large mullet and 3 small mullet tested in each container]

Container	Material in container	Distress time [1]	Death time [2]	Number (in millions)		pH [3]	Salinity [3] ‰	Remarks
				Gymnodinium brevis per liter [3]	Bacteria per ml. [3]			
No. 1	Millipore filtrate (HA membrane) of 1 liter of bacteria-free *G. brevis* culture, 3.3 million organisms per liter. Two membranes used; first one became clogged.	(0:09) 0:19 0:42 1:10	(0:14) 0:25 1:27 1:44	0		7.7	33.3	Salinity—⅔ hour after fish died.
No. 2	Paper filtrate (Whatman No. 40) of 1 liter of the same culture that was used to prepare the filtrate in container 1.	(0:46) 0:34 0:44 0:55	(0:54) 0:44 1:10 8:05	0.0080		7.5	33.0	Some *G. brevis* passed the filter paper when was inadvertently o flowed.
No. 3	Both millipore membranes used to prepare filtrate in container 1 eluted in 1 liter of sterile culture medium, which was 1 week old with a pH of 7.8.	(Not known) Not known 5:00 5:05	(8½–13½ hr.) 5:00 5:06 5:10			7.5	30.7	Salinity and pH—after hours.
No. 4	Filter-paper disc used to prepare the filtrate in container 2 eluted in 1 liter of sterile medium from same batch as used for elution in container 3.	(0:31) 0:36 0:37 0:38	(0:39) 0:42 0:42 0:42			6.8	28.9	pH—⅔ hour, salinity— hours after last fish di
No. 5	Millipore filtrate (HA membrane) of 1 liter of sterile culture medium—control for container 1.	(4) 0:48 2:12 (4)	(4) 2:01 2:17 (4)			7.2	33.2	
No. 6	Paper filtrate (Whatman No. 40) of 1 liter of sterile medium from same batch as used to prepare filtrate in container 5—control for container 2.	(4) Not known (4) (4)	(4) 1:13 (4) (4)			7.0	33.4	
No. 7	Millipore membrane used to prepare filtrate in container 5 eluted in 1 liter of sterile medium from same batch as used for elution in container 3—control for container 3.	(4) 0:41 (4) (4)	(4) 0:53 (4) (4)			7.1	32.9	
No. 8	Filter-paper disc used to prepare the filtrate in container 6 eluted in 1 liter of sterile medium from same batch as used for elution in container 3—control for container 4.	(4) (4) (4) (4)	(4) (4) (4) (4)			7.1	30.7	
No. 9	Bacteria-free *G. brevis* culture	(0:15) 0:16 0:23 0:32	(0:23) 0:42 1:45 1:54	2.4 (2.3)	0 (0.0050)	7.6	33.0	Salinity—½ hour after fish died.
No. 10	do	(0:16) 0:32 0:48 0:58	(0:28) 1:41 2:14 2:28	3.1 (2.9)	0.000008 (0.015)	7.3	32.8	
No. 11	do	(0:30) 0:42 0:43 0:43	(0:41) 0:44 0:45 1:08	2.7 (2.8)	0.000001 (0.030)	7.1	33.0	Salinity—1¾ hours af last fish died.
No. 12	do	(0:32) 0:35 0:37 0:41	(0:40) 0:45 0:45 0:45	3.3 (1.8)	0.00010 (0.0044)	7.1	33.1	Salinity—1½ hours af last fish died.
No. 13	Sterile culture medium—control for containers 9, 10, 11, and 12.	(4) 0:18 0:30 0:52	(4) 0:25 0:33 1:38		0.000002 (in excess of 80 millions)	6.6	33.3	
No. 14	do	(Not known) 0:46 0:52 Not known	(8½–13½ hr.) 0:47 0:56 2:39		0.000025 (25 to 50 millions)	5.9 (6.4)	33.3	Salinity, first pH, a second bacterial coun after 14 hours. Seco pH (6.4)—after 25 hou
No. 15 [5]	Autoclaved 85% sea water	(Not known) Not known Not known Not known	(14:10) 8½–13½ hr. 8½–13½ hr. 8½–13½ hr.		0.000012 (in excess of 80 millions)	7.3	33.0	Salinity, pH, and seco bacterial count—aft 14½ hours.
No. 16	do	(Not known) Not known (4) (4)	(8½–13½ hr.) 7:16 (4) (4)			7.6	33.1	
No. 17	10-week-old unialgal *G. brevis* culture that was not replenished with fresh medium.	(0:12) 0:24 0:54 1:48	(0:14) 0:45 0:56 2:30	2.6 (2.7)	1.3 (1.4)	7.6	33.2	
No. 18	do	(0:06) 0:12 0:14 0:24	(0:10) 0:15 0:20 0:25	2.6		7.9	33.2	pH—1 hour, salinity— hours after last fish di

[1] Time (hr.: min.) required for fish to show first signs of imbalance. The first distress time listed for each container, in parentheses, pertains to large mullet (4½ to 5½ in. long) and the other three distress times pertain to small mullet (1 to 1¼ in. long).

[2] Time (hr.: min.) of cessation of opercular movement. The first death time listed for each container, in parentheses, pertains to large mullet and the other three death times pertain to small mullet.

[3] The first *G. brevis* and bacterial counts listed for each container were obtained from samples taken from the containers before addition of the fish except for containers 17 and 18. For these containers, the sample was withdrawn from the culture before portions were dispensed into containers 17 and 18. With the exceptions noted under Remarks, samples for the second *brevis* and bacterial counts, in parentheses, as well as pH and salinity samp were collected either shortly after the last fish died if all fish died within hours or at the end of the 24-hour experimental period if at least 1 fi survived.

[4] Distress or death did not occur during the 24-hour test period.

[5] The large mullet in container 15 probably died of oxygen deficiency. T fish appeared in good condition after 13½ hours and the material in the co tainer was being aerated. When noticed again 30 minutes later the fish w dying and no air was being pumped into the container at this time.

and unialgal *G. brevis* cultures. However, large *M. cephalus* survived considerably longer in the two containers of sterile culture medium than in the *G. brevis* cultures. One large fish survived the 24-hour test period and the other lived at least 8½ hours. In contrast, the death time for the six large mullet in three different unfiltered *G. brevis* cultures, two of which were bacteria-free, varied from 10 to 41 minutes. All fish in the sea water lived for at least 7½ hours and two of them survived the test period.

The early deaths of the small mullet in containers 13 and 14 were probably due to the abnormally low pH of the culture medium. The pH of the material in container 14 was 5.9 about 4 hours after beginning of experiment and increased to 6.4 after 25 hours. A pH value of 6.6 was obtained for the culture medium in container 13 at the end of the test period.

The control fish survived much better in the batch of sterile culture medium (containers 5, 6, 7, and 8) used in the filtration phase of this experiment than they did in the batch of medium (containers 13 and 14) used in the nonfiltration phase. All (4) of the large mullet and two-thirds (8) of the small mullet subjected to filtrates and residues of culture medium survived the 24-hour test period. On the contrary, none of the fish (4 large mullet and 12 small mullet) exposed to filtrates and residues of the *G. brevis* culture survived the test period. The difference between the effects of the two methods of filtration on the toxicity of the *G. brevis* culture was marked. The millipore filtrate was much more toxic to the fish than the residues; in the filtrate the death times varied from 14 to 104 minutes in contrast with a variation of 5 to 13½ hours in the residues. The toxicity of the filter-paper residues was greater than the filtrate; the test fish lived from 39 to 42 minutes in the residues, whereas they survived from 44 minutes to about 8 hours in the filtrate.

The attempt to reduce the initial bacterial contamination of the bacteria-free *G. brevis* cultures and the sterile culture medium by the previously mentioned precautions was successful. The counts for these materials varied from 0 to 100 bacteria per ml. The bacterial counts in the initially bacteria-free *G. brevis* cultures varied from 4,400 to 30,000 per ml. at the time the last fish died in the container (¾ hour to 2½ hours later). The initial count in the unialgal culture was 1.3 million bacteria per ml.; when the last fish died 2½ hours later the count was 1.4 million bacteria per ml. At the end of the test period the bacterial concentration was in excess of 80 million per ml. in one container (13) of initially sterile culture medium. After about 14 hours the count was 25 to 50 million bacteria per ml. in the other container (14) of culture medium and in excess of 80 million per ml. in one container of sea water.

The pH of the initially sterile culture medium in container 14 increased from 5.9 to 6.4 during the 11-hour period after the last fish died. Additional fish were subjected to this material to test its toxicity at the higher pH.

EXPERIMENT 9a.—Supplementary Toxicity Tests of Some Test Materials Previously Used in Experiment 9

Near the close of experiment 9, we conducted a supplementary study to determine whether the initially sterile culture medium in container 14 was still toxic to small *M. cephalus*. Five other containers originally a part of experiment 9—one container (15) of 85-percent, autoclaved sea water and the four containers (9, 10, 11, and 12) of initially bacteria-free *G. brevis* culture—were included in this study. The other containers of culture medium (13) and sea water (16) were not used because this part of experiment 9 was still in progress, as they contained one or more live fish.

Small mullet from the group used in experiment 9 served as test fish. Four of these fish, which were maintained in 85-percent, autoclaved sea water for 24 hours, were placed in each container. Only two of the four containers of *G. brevis* culture were aerated in an attempt to determine the effects of agitation and aeration on the toxicity of *G. brevis*. The culture medium (container 14) and sea water (container 15) were not aerated.

The results (table 9) show that *G. brevis* cultures were still toxic to small mullet, but the fish survived well in the previously toxic culture medium. The 16 fish in the *G. brevis* cultures died in from 8 to 125 minutes; 10 of them survived less than 1 hour. In the control materials (containers 14 and 15), however, only 1 of the 8 fish failed to survive the 24-hour test period; 1 fish in the sea water succumbed after 5¾ hours.

TABLE 9.—*Experiment 9a: Effects on* Mugil cephalus *of certain test materials initially used in experiment 9*

[4 small fish tested in each container; see table 8 for container contents]

Container	Distress time [1]	Death time [2]	Number (in millions) of *Gymnodinium brevis* per liter	Remarks
No. 9	0:37	1:26	0.9 (sample taken 30 minutes after 4 fish were added).	Material aerated continuously during previous 24-hour period, aerated vi ously for last 12 hours and during experimental period. Material not clou
	1:02	1:28		
	1:18	2:05		
	1:50	2:05		
No. 10	0:05	0:36	2.4 (sample taken 25 minutes after 4 fish were added).	Material neither aerated during previous 21-hour period nor during exp mental period. Material not cloudy.
	0:20	0:55		
	0:20	1:11		
	0:20	1:11		
No. 11	Not known	0:08	1.3 (sample taken 15 minutes after 4 fish were added).	Material not aerated during previous 22-hour period. Vigorous aeration be 18 minutes before fish were added and continued throughout experim Material not cloudy.
	0:08	0:18		
	0:10	0:18		
	0:18	0:34		
No. 12	0:05	0:10	1.4 (sample taken 15 minutes after 4 fish were added).	Material neither aerated during previous 23-hour period not during exp mental period. Material not cloudy.
	0:05	0:10		
	0:08	0:10		
	0:08	0:13		
No. 14	[3]	[3]		Material neither aerated during previous 10-hour period nor during exp mental period. Material was cloudy when fish were added. Two developed heavy microbial growth on caudal fin; they died between 30 48 hours after study was begun. Two fish alive after 5 days. Mate relatively clear at this time.
No. 15	Not known	5:47		Material neither aerated during previous 9-hour period nor during experime period. Material cloudy when fish were added, but relatively clear 5 d later. Three fish alive after 5 days.
	[3]	[3]		
	[3]	[3]		
	[3]	[3]		

[1] Time (hr.:min.) required for fish to show first signs of imbalance.
[2] Time (hr.:min.) of cessation of opercular movement.
[3] Distress or death did not occur during the 24-hour test period.

Although the experiment was discontinued after 24 hours, containers 14 and 15 were set aside and observed for 4 more days. Two of the fish in the culture medium died between 30 and 48 hours, and the other two were alive after 5 days. The three fish remaining in the sea water also lived through 5 days. There was some indication that nonaerated *G. brevis* cultures were more toxic than the aerated.

RESULTS OF EXPERIMENTS WITH BACTERIA-FREE CULTURES

The results of the experiments with bacteria-free *G. brevis* confirm that this organism produces a fish-killing substance(s) as indicated by tests with unialgal cultures. Bacteria-free *G. brevis* was toxic to the two species of fish tested (*Cyprinodon variegatus* and *Mugil cephalus*). The minimal death time for *C. variegatus* was about 3¼ hours. The mullet were more sensitive, with a minimum death time as low as 15 minutes. Furthermore, small mullet appear to be less sensitive to the substance than the large ones, since the three small fish in each container of *G. brevis* culture outlived the large one. The concentration of *G. brevis* in these bacteria-free cultures varied from 2.3 to 4.8 million organisms per liter. Such concentrations are considerably less than the 10 to 60 million per liter sometimes encountered in areas where dead or dying fish occur.

There was good agreement between the resu of experiments 8 and 9 with regard to the toxici of bacteria-free *G. brevis* cultures to small *M cephalus*, which was the only species used in bo experiments. Nevertheless, the small mullet su vived much better in the control materials experiment 8 than in experiment 9. In the latt experiment, the small mullet died rapidly in o particular batch of sterile culture medium (co tainers 13 and 14). Large *M. cephalus* in th batch of medium, however, survived much bett than the small mullet.

We attribute the early death of the small mull in the culture medium placed in containers 13 a 14 of experiment 9 to the abnormally low pH this particular batch of medium. Moreover, th relatively poor survival of these fish in some of th other control containers of experiment 9 was po sibly due to their being damaged by the vigoro thrashing about of the large mullet. The leng of some of the large test fish slightly exceeded th inside diameter of the experimental containers.

The low pH of the material in container 14 sug gests that the batch of medium placed in contain 14 was abnormal before the fish were added. A proximately 14 hours after experiment 9 bega the pH of the medium in container 14 was 5.9 an the bacterial count was between 25 and 50 millio per ml. Since the pH of this material increase from 5.9 to 6.4 during the next 11 hours in spite o

heavy bacterial growth, the initial pH of this particular batch of sterile medium possibly was lower than 5.9. The pH of the medium in container 13 (duplicate for container 14) was 6.6 after 24 hours. The pH values, after 24 hours, for the millipore and paper filtrates of the other batch of sterile culture medium used in experiment 9 were 7.2 and 7.0, respectively. The initial pH of month-old sterile culture medium and bacteria-free *G. brevis* cultures used in experiment 8 varied from 7.6 to 7.7. No pH values were ascertained initially in experiment 9, excepting a pH of 7.8 for the week-old sterile culture medium used for eluting the millipore and filter-paper residues.

The pH of culture medium either with or without *G. brevis* customarily drops after fish are introduced; this decrease probably results from increased bacterial growth and accumulation of fish waste products. Excepting two containers (13 and 14) of culture medium and a container (4) of filter-paper residues of a bacteria-free *G. brevis* culture eluted in sterile culture medium in experiment 9, a minimal pH value of 7.0 was recorded after test periods as long as 24 to 30 hours in the two experiments, 8 and 9, conducted with initially bacteria-free *G. brevis* cultures and sterile culture medium. Small mullet survived in culture medium with pH values as low as 7.0 and 7.1 in experiments 8 and 9.

We do not know why the pH of one particular flask of culture medium used in experiment 9 was so low. It is our surmise that the abnormally low pH resulted from failure to rinse the culture flask after 7-percent, hot nitric acid was used in the cleaning process. By actual test we found that failing to rinse the flask after the nitric-acid treatment did significantly lower the pH of 2.0 liters of sea-water-base medium. In this particular test the pH of the autoclaved medium stabilized at approximately 6.4.

A flask of culture medium, companion to one with the low pH (containers 13 and 14) of experiment 9, that was inoculated with *G. brevis* failed to support growth of this organism after incubating for 1 month. Unfortunately, the medium in this flask was discarded without checking the pH. Since the two flasks of medium were prepared at the same time, we consider that *G. brevis* possibly failed to grow because the pH was unfavorable. We have experienced thus far only this one failure of bacteria-free *G. brevis* to grow in low-form culture flasks (3-liter). Growth of *G. brevis* has not occurred in sea-water-base medium with an initial pH of less than 7.0. Furthermore, preliminary studies indicate that a pH below 7.4 is unsatisfactory for this organism.

The results of experiment 9a (table 9) show that the culture medium in container 14 was no longer toxic to small mullet when retested about 24 hours after experiment 9 began. The pH of this medium was 6.4 about 1 hour after start of experiment 9a. All four test fish were alive after 24 hours; two of them were alive after 5 days. The pH of the medium in container 14 was 6.9 at this time.

The effects of millipore and paper filtration on the toxicity of bacteria-free *G. brevis* cultures were the same as observed when unialgal cultures were so treated. The more toxic portion of the culture passes through the millipore membrane, whereas filter paper retains the more toxic fraction.

Bacteria-free *G. brevis* cultures proved just as toxic as the unialgal ones in simultaneous tests with comparable concentrations of this organism. For example, in experiment 8 (table 6) the death times for *M. cephalus* in initially bacteria-free cultures varied from a minimum of 15 minutes to a maximum of 4 hours and 44 minutes. Seven of the 16 *M. cephalus* died in less time than was required (1 hour and 13 minutes) to kill the two *M. cephalus* subjected to a unialgal culture. The *C. variegatus* succumbed in the bacteria-free cultures in periods varying from a minimum of 3 hours and 18 minutes to a maximum of 32 hours; 13 of the 16 test fish died within 8 hours. Nine of the 16 *C. variegatus* in bacteria-free cultures died in less than the minimum time (6¼ hours) required to kill the two *C. variegatus* in a unialgal culture. Further data for comparison of the effects of bacteria-free and unialgal cultures are available from experiment 9 (table 8). Two large *M. cephalus* subjected to unialgal cultures died in 10 and 14 minutes. The four large mullet in the bacteria-free cultures died within 23 to 41 minutes. The death times for six small *M. cephalus* in the unialgal cultures varied from 15 minutes to 2½ hours. The extremes of death times for the 12 small mullet in bacteria-free cultures were quite similar—25 minutes to 2 hours and 28 minutes.

Despite the evidence that bacteria are not directly responsible for the toxic effects of *G. brevis* cultures, the possibility that bacteria play a

significant role in the development of *G. brevis* blooms should not be overlooked. Such organisms may contribute appreciably to the nutrition of *G. brevis*. For example, some of the bacteria isolated from unialgal *G. brevis* cultures produce vitamin B_{12}-active substances (Starr et al., 1957). Vitamin B_{12} apparently stimulates the growth of *G. brevis* in sea-water-base medium (Wilson and Collier, 1955).

Once fish kills are initiated by blooms of *G. brevis*, excessive bacterial growth resulting from the increased availability of organic matter may possibly cause the blooms to decline in isolated situations. Bacteria could unfavorably affect *G. brevis* blooms by producing substances toxic t this organism, by competing for nutritive sul stances, and by adversely altering the pH. W have frequently observed the failure of unialga *G. brevis* to grow in tubes in which the mediu became cloudy with bacterial growth.

Another role for which bacteria must be con sidered is that of a detoxicating agent. Shilo an Aschner (1953) found that bacteria decreased th toxicity of cultures of *Prymnesium parvum*, marine and brackish water chrysomonad that toxic to fish. Similarly, bacterial activity ma influence the toxicity of *G. brevis* in the laborator and in nature.

TOXICITY STUDIES WITH BACTERIA

Although the available evidence indicated that *Gymnodinium brevis* causes the toxicity of unialgal cultures, direct proof was lacking prior to the development of bacteria-free cultures. In addition to the bacterial studies previously considered, the possibility that bacteria may cause all or some of the toxic effects was investigated by testing pure cultures of some of the bacteria from unialgal *G. brevis* cultures. Furthermore, toxicity tests were conducted with pure cultures of an unidentified red-pigment-producing bacterium isolated from Florida water and *Flavobacterium piscicida* Bein. Bein (1954) suggested that *F. piscicida*, a chromogenic bacterium, possibly was a cause of mass fish mortality associated with discolored water off the west coast of Florida.

BACTERIA ISOLATED FROM UNIALGAL *GYMNODINIUM BREVIS* CULTURES

Because of the preliminary nature of these studies and the crudeness of the quantitative bacterial estimates, only a summary will be presented. The bacteria used have not been identified; presently they are being characterized morphologically and physiologically by Dr. T. J. Starr. The test fish were Gulf killifish (*Fundulus similis*).

Two test fish per container (1-liter beaker) were subjected to about 500 ml. of nonaerated test materials. Bacterial suspensions were prepared by adding 16.5 ml. of a 24-hour culture to 500 ml. of filtered Galveston Bay water. Control materials consisted of the same ratio of sterile culture medium and bay water as well as bay water alone. Crude estimates of bacterial concentrations were made by preparing a pour-plate of 0.02 to 0.0 ml. of a sample collected shortly after the fis were added. A second sample was taken eithe after both fish died or after 23 hours if at least on fish survived this period. The colonies were to numerous to count in most of the plates prepare from the second set of samples. Therefore, no even rough estimates could be made for th bacterial concentrations.

The most-abundant colony type isolated fro unialgal *G. brevis* cultures on Spencer's pepton sea-water agar and Bein's peptone agar is gener ally a convex, white, opaque colony produced b Gram-negative rods. This colony type ma represent several different species and physiologi cal types of bacteria. Two separate isolates o the white, opaque colony did not give evidence o being toxic to *F. similis*. The initial bacteria concentration was in the order of 0.5 to 1 millio per ml.

A flat, white, transluscent colony with an irides cent sheen, also produced by Gram-negative rods is usually the second most-abundant colony typ isolated from unialgal *G. brevis* cultures. A initial concentration of approximately 1 millio bacteria per ml. of this colony type gave n evidence of toxicity to *F. similis*.

Chromogenic bacteria constitute only a smal portion of the bacterial flora of unialgal culture of *G. brevis;* however, yellow-pigment-producing bacteria become abundant in cultures treated with dihydrostreptomycin sulfate. They dominate i *G. brevis* cultures treated with 500 to 1,000 μg. o this antibiotic per ml., and often occur in nearly pure culture. This antibiotic may enhance th

growth of the "yellow bacteria" by inhibiting competing bacteria. Dihydrostreptomycin sulfate (125 μg. per ml.) initially lowers the pH of the culture medium by 0.5 to 0.8 of a pH unit. This change in the medium may be a factor favoring the increased growth of the pigment-producing bacteria.

Cultures of an isolate from a nontreated and a dihydrostreptomycin-treated unialgal culture, each with an initial count of about 1 million yellow bacteria per ml., had no toxic effects on *F. similis*. Plates prepared from samples taken 23 hours after the start of the experiment showed no yellow colonies. The counts of all bacteria were about the same in the initial and 23-hour samples.

A CHROMOGENIC BACTERIUM ISOLATED FROM THE WEST COAST OF FLORIDA

After Bein (1954) reported the toxic effects of *Flavobacterium piscicida* to fish, we made a cursory check for chromogenic bacteria in Florida off the Fort Myers-Naples area during November 1954. *Gymnodinium brevis* was present in the area at that time although the maximum concentrations were usually less than 1 million organisms per liter. Small fish kills, mainly of mullet, were being reported sporadically at that time. During the sampling trips, however, we observed less than 10 dead fish. Surface samples from 15 stations in this area were plated on Spencer's sea-water peptone agar. Pour-plates containing 1 ml. of each sample were prepared within 1 minute after collection to avoid possible changes in the bacterial flora. All except two of the samples from the 15 stations contained *G. brevis*, and the counts varied from 7,000 to 0.5 million per liter.

A white, opaque colony was the most abundant type in the 15 samples; some samples showed a few lemon-yellow colonies. A total of two red colonies were observed in the 15 plates. One of these colonies was isolated from a sample taken 5 miles west of Wiggin's Pass on November 4, 1954. The *G. brevis* count for this sample was 8,000 per liter. The red-pigment-producing bacterium, which has not been identified, is a Gram-negative, motile rod.

A 24-hour pure culture of the "red bacterium" was tested for toxicity to *Fundulus similis* as a part of the studies dealing with bacteria isolated from unialgal *G. brevis* cultures. Two fish were tested in each of the four containers of test material in which the bacterial count varied from approximately 0.5 to 1 million per ml. The bacterial culture gave a pink tint to the test materials. All eight fish died within 2 to 8 hours. After the last fish died in each of the four containers, samples were taken for bacterial counts. The red colonies in the plates prepared with 0.02 to 0.03 ml. of these samples were so abundant that enumeration was impossible. We believe that the minimum concentrations were of the order of 1 to 2 million red bacteria per ml. at the time the last fish died.

A 6-month-old unialgal *G. brevis* culture (replenished with fresh medium about three times weekly) that contained 1.3 million organisms per liter killed the test fish less rapidly than cultures of the red bacterium. In the *G. brevis* culture, one fish died after 7½ hours and the other one died after 10 to 19 hours. These death times appear relatively long when compared with the usual death times of fish subjected to other unialgal cultures. These results may mean either that *F. similis* is less sensitive to unialgal cultures than other fish tested thus far, or that this culture was less toxic than the others.

FLAVOBACTERIUM PISCICIDA BEIN

A chromogenic bacterium was isolated by Reuben Lasker (Bein 1954) from a pooled water sample collected after the occurrence of a fish kill associated with discolored water in Whitewater Bay on the southwest tip of Florida. Bein found that 24-hour cultures of this bacterium, which he named *Flavobacterium piscicida*, killed several species of marine fish. He gave no quantitative values concerning the concentration of bacteria used other than that 500 ml. of a 24-hour culture of this species grown in a 0.1-percent peptone solution in aged sea water were added to 4 gallons of continuously aerated sea water. After 24 hours all fish in the experimental aquariums died and the water exhibited a bright orange-yellow discoloration.

We attempted to estimate the minimum number of *F. piscicida* required to kill mullet (*Mugil cephalus*), since we desired to know what concentration of this bacterium might be required to kill fish under natural conditions. The Marine Laboratory of the University of Miami provided the stock from which our cultures were derived. Since Bein gave no indication of the amount of inoculum used to seed the medium to obtain the

24-hour test cultures, we could not duplicate his inoculation procedures.

In our experiment, 24-hour cultures were obtained by inoculating two loops of culture removed from a 24-hour slant culture (1-percent peptone agar) into duplicate Erlenmeyer flasks containing approximately 150 ml. of sterile 0.1-percent peptone solution in aged sea water. One flask was incubated at 30° C. and the other at 25°. The culture incubated at 30° was deep orange after 24 hours and the one incubated at the lower temperature was yellow. The more-intensely pigmented culture was used because it probably contained the greater concentration of bacteria. The ratio of bacterial-culture volume to sea-water volume varied from a maximum of 33.0 ml. of 24-hour culture to 1.0 liter of sea water, which is about the ratio used by Bein, to a minimum of one-hundredth of this ratio. Sea water and sterile 0.1-percent peptone solution were used as control materials. Each experimental container (2-liter beaker) received 1.0 liter of sea water, which was aerated continuously. Two mullet (3 to 4 in. long) were placed in each container. The test fish, which had been maintained in the laboratory for several days, were acclimated in the experimental containers for about 24 hours before the beginning of the study. The water, which became cloudy in all containers by the end of the acclimation period, was replaced with fresh sea water shortly before the test materials were added. Samples were collected for bacterial counts about 30 minutes after the experiment began. These samples were refrigerated ½ hour to 2 hours before preparation of the pour-plates.

All fish were alive when the experiment was discontinued 5 days later and had shown no evidence of distress. A second set of bacterial count samples was taken at this time. These samples were plated after being refrigerated ½ to 3 hours. All bacterial plates were prepared with Bein's agar medium, and they were counted after 6, 12, 15, and 21 days' incubation. The number of pigmented subsurface colonies as well as the pigment intensity of such colonies increased after prolonged incubation. After 21 days, however, some colonies began to lose pigment. Bacterial counts of the test materials used in this experiment are listed in table 10.

RESULTS OF STUDIES WITH BACTERIA

The results of the preliminary experiment with some of the bacteria isolated from unialgal *Gymnodinium brevis* cultures suggest that cultures of the two dominant colony types, both nonpigmented, and a sparsely occurring pigmented form are not toxic to fish.

On the contrary, a "red bacterium" isolated from *G. brevis*-infested water off the west coast of Florida appears to be toxic to fish. Nevertheless, until this bacterium is found more abundantly or its association with fish kills is established, we shall not consider it of importance as a cause of fish mortality occurring off the west coast of Florida. Further toxicity studies with this organism have been discontinued until such

TABLE 10.—*Initial and final counts of bacteria in materials used in experiment testing effect of* Flavobacterium piscicida *on* Mugil cephalus

Container	Material in container (in addition to 1.0 liter of sea water)	Initial count (in millions)		Final count (in millions) after 5 days	
		Bacteria per ml.	*Flavobacterium piscicida* per ml.[1]	Bacteria per ml.	Orange and yellow-pigment-producing bacteria per ml.[2]
No. 1	None	[3] 0.80	None	1.6	0.05
No. 2	33.0 ml. sterile 0.1% peptone solution	[4] 0.46	None	1.7	0.06
No. 3	33.0 ml. 24-hour *F. piscicida* culture	5.0	1.9	3.6	0.0
No. 4	16.5 ml. 24-hour *F. piscicida* culture and 16.5 ml. sterile 0.1% peptone solution	3.2	0.80	3.1	0.02
No. 5	8.2 ml. 24-hour *F. piscicida* culture and 24.8 ml. sterile 0.1% peptone solution	1.2	0.42	2.5	0.04
No. 6	do	1.2	0.35	6.7	0.03
No. 7	3.3 ml. 24-hour *F. piscicida* culture and 29.7 ml. sterile 0.1% peptone solution	0.40	0.22	1.8	0.03
No. 8	0.3 ml. 24-hour *F. piscicida* culture and 32.7 ml. sterile 0.1% peptone solution	0.32	0.020	3.7	0.02

[1] The number of *F. piscicida* based on the observed number of deep-orange colonies.

[2] Since some of the chromogenic colonies varied from either yellow to orange or orange to yellow at various intervals during the 21 days of incubation, orange and yellow colonies were combined to indicate the maximum observed number of bacteria in this color range. There were no definitely orange colonies in samples from containers 3 and 8; about one-third of the pigmented colonies in the sample from container 6 and more than one-half of those in samples from all other containers were definitely orange.

[3] Approximately one-sixteenth of the bacteria produced yellow pigment.

[4] Approximately one-tenth of the bacteria produced yellow to light-orange pigment.

me that evidence is obtained to implicate it as fish-killing agent in nature.

A bacterium producing red pigment was isolated y Bein (1954) from Indian River on the east ast of Florida at the time of an outbreak of dis-lored water during August 1951. Although the ater was discolored when the isolation was made, o fish either dead or alive were observed. Bein und that this bacterium was nontoxic to several ecies of fish. Howell (1953), studying the same tbreak of discolored water, reported that no eat quantity of fish was killed; and that the dis-loration was caused by a dinoflagellate, which e described as a new species, *Gonyaulax monilata*.

The attempt to determine the minimal lethal ncentration of *Flavobacterium piscicida* failed, nce none of the fish died during the experi-ental period. The reason for this lack of toxic fect is not known. Although we followed as early as possible the procedures used by Bein 954), the experimental conditions employed ere unfavorable for the bacterium, especially ith regard to toxin and pigment production. he bacterial counts given in table 10 indicate at high concentrations of *F. piscicida* (orange-igment-producing bacteria) were present ini-ally. The second counts, which were made om samples collected at the end of the 5-day xperimental period, however, showed that either is bacterium or its chromogenic characteristic ecreased greatly during the intervening period. similar disappearance of chromogenic bacteria ccurred during the toxicity study with yellow acteria isolated from the unialgal *G. brevis* ultures. The experimental conditions may have ffected the toxicogenic and chromogenic char-cteristics of this bacterium in several possible ays: (1) By killing the organism, (2) by in-ibiting the growth of the organism, or (3) by ltering the toxicogenic and chromogenic prop-rties of the organism.

With only initial and terminal counts available, he question as to when the decline of *F. piscicida* ccurred—either early or gradually during the xperimental period—can not be answered. At o time during the 5-day period did the contents f any container show the bright orange-yellow iscoloration observed by Bein after 24 hours. he contents of container 3, which received the naximum amount of *F. piscicida* culture, ex-ibited a tinge of orange after 24 hours. This slight discoloration gradually became less notice-able and completely disappeared by the fifth day.

The numbers of *F. piscicida* recorded in table 10 represent minimal counts, which may be considerably lower than the actual concentrations, because several of the white subsurface colonies produced the characteristic orange pigment after being transferred to agar slants (1-percent pep-tone). All deep-orange colonies in plates prepared with samples that contained *F. piscicida* were considered to have been produced by this species. A few orange colonies appeared in plates prepared from some of the control samples, particularly in those taken after 5 days. We made no allowance for orange colonies which possibly were not produced by *F. piscicida*, since the counts were considered low even with the inclusion of such colonies. An examination of the initial counts (table 10) obtained for the control containers (1 and 2) and the two containers (7 and 8) receiving the smallest volumes of bacterial cultures suggests that counts of bacteria, exclusive of *F. piscicida*, varied in the order of 0.3 to 0.8 million per ml. From these values we presume that the actual concentration of *F. piscicida* was approximately twice as great as the values listed. For example, the initial number of *F. piscicida* in container 3 was probably about 4 million if one assumes that only about 1 million of the bacteria per ml. were other than *F. piscicida*. The initial counts of this bacterium were in relatively good agreement with the various dilutions employed. For example, the material in container 3, which received the greatest volume of bacterial culture, yielded a count of about 2 million chromogenic bacteria per ml., whereas the material in container 8, which received only one-hundredth as much as container 3, gave a count of 20,000 chromogens per ml.

Since Bein gave only the ratio of *F. piscicida* culture and sea water employed, the concentrations of bacteria used in his studies cannot be directly compared with those we used. If the amount of water discoloration is proportional to the abundance of *F. piscicida*, we presume that the water in Bein's aquariums probably contained in excess of 4 million *F. piscicida* per ml. at the end of the 24-hour experimental period. This presumption is based on the observation that the sea water in container 3 of our experiment was not appreciably

discolored after the addition of 2 to 4 million *F. piscicida* per ml., whereas in Bein's studies the water was bright orange-yellow 24 hou after it received the bacterial cultures.

GENERAL DISCUSSION

The well-established association of *Gymnodinium brevis* with the sporadic mass mortality of fish and other marine animals that has occurred in the Gulf of Mexico since 1947, in conjunction with the clear-cut laboratory demonstration that this dinoflagellate in pure culture is toxic to fish, leaves no reasonable doubt that this organism causes these mortalities. Considering this evidence, we propose the name "brevis red tide" for such mortalities instead of the nonspecific term "red tide" that is used commonly in popular and scientific writing. In our opinion, ample characteristics properly identify these mass mortalities occurring in the Gulf of Mexico as a distinct phenomenon. This phenomenon can be diagnosed by the presence of *G. brevis* in the waters in which fish and other marine animals are dying. An additional diagnostic characteristic is the odorless, human-respiratory-tract irritant often present in or near mortality areas where droplets of sea water become air-borne as a result of wind, wave action, et cetera (Galtsoff 1948; Gunter et al., 1948; Woodcock 1948; Ingle 1954; and others).

Failure to find either one or both of the mentioned diagnostic characteristics in an isolated area of dead or dying fish would not necessarily eliminate *G. brevis* as the cause. At least four possible reasons support this statement: (1) The conditions or agents required to make droplets of sea water air-borne may be absent. (2) Dead fish may drift or be carried into an area either unsuitable for the survival of *G. brevis* or removed from the bloom. (3) An isolated mass of water in which *G. brevis* is blooming may suddenly become unsuitable for this organism and yet not lose its toxicity to fish until sometime later. Experimental evidence supports this suggestion, since the removal of living *G. brevis* from cultures by millipore filtration or the killing of the organisms with gentle heat did not inactivate the toxic substance. A specific diagnostic test for the toxic substance(s) produced by this organism would be helpful in diagnosing the cause of mortality in such cases. *G. brevis* is so delicate that under adverse conditions it may die within a matter of minutes, leaving only fragmentary remains that are not readily identifiable. (4) The fish may contact t toxic substance in a bloom of *G. brevis* in one ar and yet not succumb until it moves into an ar where the organism is not flourishing. This pos bility is based on limited observations that fis exposed to *G. brevis* cultures for short periods a removed before they showed distress, died aft being placed in sea water. The death time aft removal to sea water appears to decrease as t exposure time is increased.

The results of our studies support Galtsof (1948) conclusion that fish are not killed by cloggi of the gill filaments by masses of *G. brevis*. T available evidence makes untenable the view th fish suffocate as a result of mechanical occlusi of gill surfaces by the mere presence of large nu bers of organisms. Results of the studies wi both heated and filtered *G. brevis* cultures, tes with other dinoflagellate cultures, and oxyg analyses of *G. brevis* cultures emphasize the exis ence of a toxic substance(s). *G. brevis* cultu heated to 35° and 45° C. did not lose their toxici although the organisms were disrupted. Lik wise, the removal of this organism (both unialg and bacteria-free) by millipore filtration did n detoxicate the cultures. Unialgal cultures of *splendens* and *Prorocentrum* sp. were nonto despite the fact that the number of organis compared to and even, in some cases, exceed the concentrations in the toxic *G. brevis* cultures

Filtrates of *G. brevis* cultures are toxic; ho ever, the method of filtration determines wheth the more toxic portion of the culture will pa through or be retained by the filter membra In our studies conducted with both unialgal a bacteria-free cultures, the filter-paper residu eluted in either sea water or culture medium we more toxic to the fish than were the filtrates. T results were reversed when a culture was pass through a millipore membrane under suctio the more toxic portion passed through the me brane. The reasons for the different effects these two methods of filtration are not know The filter materials differ in composition and siz the millipore membrane (diameter 47 mm.) made of cellulose esters, whereas the filter pap

diameter 18.5 cm.) consists of cellulose fibers. Retention of the toxic substance by filter paper may be due to greater adsorptive area and/or to differences in physical and chemical properties. Another possibility is that filtration by gravity flow used with filter paper may result in the retention of considerably more intact organisms than in the case of millipore filtration under suction. Assuming that greater numbers of *G. brevis* were broken up by millipore filtration, more toxin might be released in such a case. However, our preliminary studies showed no apparent increase in the toxicity of *G. brevis* cultures in which the organisms were cytolyzed by gentle heating.

There are no indications that the fish kills caused by *G. brevis* result from the great masses of this organism depleting the oxygen in the sea water. Connell and Cross (1950) suggested that anaerobic conditions created by the high biochemical oxygen demand of an armored dinoflagellate, *Gonyaulax*, was the cause of mass mortality of fish associated with discolored water in Offatts Bayou (Galveston Bay) during the summer of 1949. Gunter et al. (1948) concluded that the 1946–47 incidence of mass mortality of marine animals on the west coast of Florida was not associated with low oxygen. Oxygen deficiency can be excluded as a factor in the death of the fish in the *G. brevis* cultures that were aerated. In experiment 8 (tables 6 and 7), the dissolved-oxygen content of all aerated test materials exceeded 75-percent saturation and some were as high as 90 percent. With continuous gentle aeration one of the *G. brevis* cultures was about 90-percent saturated, although the bacterial count was 24 million per ml. at the time the dissolved-oxygen content was determined.

The results of an attempt to determine the effects of aeration on the toxicity of bacteria-free *G. brevis* cultures were contradictory. In experiment 8, the fish—especially *Mugil cephalus*—showed distress and died more rapidly in the aliquot that was aerated (dissolved oxygen, 89-percent saturation) than in the one that was not aerated (dissolved oxygen, 45-percent saturation). The fish in another nonaerated aliquot (dissolved oxygen, 57-percent saturation) in this experiment showed distress much sooner than those in the aerated culture (dissolved oxygen, 82-percent saturation). In experiment 9a (table 9), *M. cephalus* died somewhat faster in the nonaerated aliquot of a *G. brevis* culture than in the aerated aliquot. It is apparent from these contradictory results that the influence of such factors as aeration cannot be evaluated until standardization of the toxicity tests is more complete.

Several factors probably influence the degree of toxicity of *G. brevis* cultures. Aside from the concentration of *G. brevis* other factors such as the growth phase of culture, pH of culture during growth and during test period, temperature and salinity of test culture, size and number of test fish, volume and degree of aeration of test culture, and bacterial growth, are among those that must be considered in standardizing the toxicity tests. Shilo and Aschner (1953) found that a number of factors influenced the toxicity of *Prymnesium parvum* cultures. The toxicity was decreased by oxidizing agents, aeration, adsorbents including pond-bottom soils, bacterial growth, and low pH (below 7.5). They improved the standardization of their toxicity tests by using a buffer to control pH of the test culture and streptomycin to suppress bacterial growth. Furthermore, the test cultures were not aerated. McLaughlin [3] working with the same organism reported that cultures grown in an alkaline medium were more toxic than those grown in an acid medium. *P. parvum* cultures (grown in alkaline media), rendered nontoxic by lowering the pH to 6.0, regain their toxicity when made alkaline (Shilo and Aschner, 1953; McLaughlin [3]).

Some of the factors mentioned above may account for the variable death times obtained in duplicate containers of *G. brevis* cultures. Since on some occasions the pattern of response in one of the duplicate containers was different from that in the other, we consider that factors other than variations of the individual test fish were responsible. For example, in experiment 6 (table 4) the fish died in 9 and 16 minutes in one container whereas death occurred after 1⅔ and 2 hours' exposure in another. Both containers (nonaerated) held similar amounts of the same unialgal culture.

There are other anomalies that defy explanation at present. One of these concerns the variation in the response of fish subjected to cultures supposedly grown under the same conditions, and

[3] McLaughlin, John J. A. The physiology and nutritional requirements of some chrysomonads. 65 pp. Thesis, Ph. D., New York University. 1956.

treated in the same manner throughout the study. For example, in experiment 8 (tables 6 and 7), container 9 received one of the duplicate pure cultures and container 10 received the other. The *G. brevis* counts of the material in the two containers and such measured factors as pH, dissolved oxygen, and salinity were comparable.

The similarity between the two containers is further emphasized by the distress times for *Mugil cephalus*—10 and 12 minutes in container 9, and 13 minutes for each of the two fish in container 10. Despite all these similarities, *M. cephalus* in container 9 died in 15 and 25 minutes in contrast with 69 and 72 minutes in container 10. However, *Cyprinodon variegatus*, a less sensitive fish than *M. cephalus*, showed more similarity of death times: 3 hours 51 minutes and 4 hours 58 minutes in container 9, and 3 hours 44 minutes and 5 hours 54 minutes in container 10.

Another anomaly in experiment 8 is the case in which one pure culture (container 12) with a *G. brevis* count of 3.4 million per liter was less toxic to both *M. cephalus* and *C. variegatus* than the duplicate culture (container 13) in which the count was 2.3 million organisms per liter. The distress times (about 4¼ hours) and death times (about 4½ hours) for *M. cephalus* in the more-concentrated *G. brevis* culture (container 12) were approximately 2 hours greater than such times in the less-concentrated duplicate culture (container 13). The distress times and death times for *C. variegatus* in each culture were not as uniform as in the case of *M. cephalus*. Nevertheless, they show that the culture in container 13 was more toxic to *C. variegatus* than the one in container 12: the fish died after about 8 and 15 hours in the former container and after about 27 and 32 hours in the latter.

We realize that due to variations in the condition of the individual test fish some will survive longer than others when subjected to toxic agents. It is our opinion, however, that the over-all uniformity in the response of the test fish within each individual container, especially in experiment 8, is a strong indication of other subtle variables of which we have no knowledge.

Despite the evidence that our toxicity studies require more standardizing, the results of all experiments reported here indicate that the sensitivity of fish to *G. brevis* cultures is variable. Our tests included six species of fish as follows: *Membras vagrans, Mugil cephalus, Fundulus gra dis, Mollienisia latipinna, Fundulus similis,* a *Cyprinodon variegatus.* Possibly the most sen tive of these fish is *M. vagrans;* the only individu tested died in 4 minutes. *M. cephalus,* t species used in the greatest number of experimen (5), showed death times varying from a minimu of 8 minutes to a maximum of 4¾ hours. T great majority of them died within an hou Small *M. cephalus* (1 to 1¼ in. long) are possib slightly less sensitive than large *M. cephal* (4½ to 5½ in. long) to *G. brevis* cultures. Th possibility is suggested by results of experiment (table 8); the large *M. cephalus* in each contain of unfiltered *G. brevis* culture, without exceptio died before any of the three accompanying sma *M. cephalus.* In some cases the first small mull died within 3 to 5 minutes after the large mulle in other cases the first small mullet died 20 to minutes later. *F. grandis* showed about the sam degree of sensitivity as *M. cephalus*—minimu death time 9 minutes, maximum death tim 2 hours and 10 minutes. The *M. latipinna* die in a minimum of 47 minutes and a maximum 85 minutes. *C. variegatus* is probably the lea sensitive of the six species tested. Its minimu death time was about 2⅓ hours, the maximum w 32 hours. The sensitivity of *F. similis* is possib comparable to that of *C. variegatus.* Two *similis* died in 7½ and 10 to 19 hours.

A chromogenic bacterium, *Flavobacterium pi cicida,* has been suggested as a possible cause the mass fish kills and associated sea-water di colorations occurring along the west coast Florida (Bein 1954). Bein found that this ba terium was toxic to several species of fish althoug he did not indicate the bacterial concentration employed. In our tests *Mugil cephalus* were no affected by initial concentrations of 2 million more *F. piscicida* per ml. Contrary to Bein experience, *F. piscicida* apparently did not gro in our experiment and possibly lived only a sho time after being added to the test medium (se water). We could scarcely detect this bacterium at the end of the 5-day test period. A "re bacterium" that we isolated from *G. brevis*–in fested water off the west coast of Florida appear to be toxic to fish. Concentrations of the orde of 0.5 to 2 million bacteria per ml. were toxic t *Fundulus similis.* The "red bacterium" has no

been encountered in sufficient abundance to implicate it as a fish-killing agent. Thus far, neither the association of chromogenic bacteria with extensive fish kills nor the natural existence of toxic concentrations of such bacteria has been established. On the other hand, *Gymnodinium brevis* has been clearly implicated on both these counts.

SUMMARY AND CONCLUSIONS

1. Since 1947, blooms of the dinoflagellate, *Gymnodinium brevis*, have been associated with sporadic mass mortalities of marine animals and discolored water in the Gulf of Mexico. Extensive laboratory studies conducted with unialgal and bacteria-free cultures, as well as related bacterial studies, offer overwhelming evidence that blooms of this organism are the direct cause of the associated mortalities.

2. Bacteria-free cultures of *G. brevis* with concentrations varying from 2.3 to 4.8 million organisms per liter were toxic to two species of test fish. Five species of fish were killed when subjected to unialgal *G. brevis* cultures containing 0.6 to 2.1 million organisms per liter. The numbers of *G. brevis* in areas of natural fish kills often greatly exceed these toxic laboratory concentrations.

3. Bacteria apparently do not produce or directly contribute to the production of the toxic substance present in *G. brevis* cultures. Bacteria-free cultures were just as toxic to fish as the unialgal ones.

4. The toxicity of *G. brevis* does not depend on the presence of the living organisms. Removing the organisms from culture by millipore filtration or killing them with gentle heat did not appear to alter the toxicity. The high dissolved-oxygen content of aerated *G. brevis* cultures eliminates oxygen deficiency as a factor.

5. The toxic substance produced by *G. brevis* readily passes through a millipore membrane, but for the most part it is retained by filter paper.

6. Studies with bacteria-free and unialgal cultures indicate that the six species of test fish are differentially sensitive to *G. brevis* cultures. The test fish, listed in order of decreasing sensitivity, were *Membras vagrans*, *Mugil cephalus*, *Fundulus grandis*, *Mollienisia latipinna*, *Fundulus similis*, and *Cyprinodon variegatus*.

7. Some chromogenic bacteria isolated from the Gulf of Mexico or adjoining bays are toxic to fish in laboratory tests. However, association of such bacteria with mass fish kills in the Gulf of Mexico has not been established. *Flavobacterium piscicida*, previously found to be toxic to several species of fish in undetermined concentrations, was not toxic to *Mugil cephalus* at an initial concentration of about 2 million organisms per ml. A red bacterium isolated from *G. brevis*–infested waters appears to be toxic to fish at concentrations in the order of 0.5 to 2 million per ml. This bacterium was uncommon during our survey since only two colonies were obtained from 15 samples of 1.0 ml. each.

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